PLASMA CONVERTING ENZYME ACTIVITY DURING THE DEVELOPMENT AND MAINTENANCE OF EXPERIMENTAL AND SPONTANEOUS HYPERTENSION IN RATS

A THESIS

SUBMITTED TO THE FACULTY OF ATLANTA UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

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DEPARTMENT OF BIOLOGY

ATLANTA, GEORGIA

DECEMBER 1976

R = X; T = 82

ABSTRACT

BIOLOGY

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Plasma Converting Enzyme Activity During the Development and Maintenance of Experimental and Spontaneous Hypertension in Rats

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Doctor of Philosophy degree conferred May 16, 1977

Thesis dated December 1976

The precise identification of the form of angiotensin recovered from in vitro incubation for plasma renin activity (PRA) determinations has become increasingly important with chemical means for quantitation such as radioimmunoassay. The ability to discriminate A I and A II quantitatively in mixtures of these peptides also provides means for quantitative assessment of angiotensin converting enzyme activity. Consequently, measurement of the ratio of A I and A II recovered as the 37 C incubation product in plasma by radioimmunoassay has been used to examine plasma converting enzyme activity (PCEA).

The antibody capture method was used for the determination of A II concentrations in plasma from 3 experimentally induced hypertensive models of male Sprague-Dawley rats. This method provides for the capture of A II formed by the action of the plasma converting enzyme by use of an excess of A II antiserum during the 37 C incubation process. Simultaneous measurements of A I concentrations which demonstrates PRA's were also determined. Whenever an increase or decrease in PRA was observed,

similar activities were demonstrated by the plasma converting enzyme.

As blood pressure increased in one model of hypertensive rats that had the left renal artery constricted with simultaneous removal of the right kidney, an increase in both PRA and PCEA was observed. Increases in these enzymatic activities, apparently caused by renal artery constriction, are assumed to be due to a negative sodium balance. Sodium depletion of this type is believed to be due to an excessive loss of sodium from the circulating blood by the kidney tubules. These physiologic phenomena apparently result in increased renal enzymatic activities. In contrast to these findings, similar PRA and PCEA activities were observed in one kidney nephrectomized control rats at 10 days. However, decreases in PRA and PCEA at 20 and 30 days indicate that some animals with only one functional kidney presumably have the ability to auto-regulate, and thereby maintain homeostasis. These results indicate an active physiological role of the converting enzyme in the maintenance of blood pressure. Increases or decreases in PCEA result in either increased or decreased concentrations of A II, the vasoconstrictor hormone of the renin-angiotensin system.

The same enzymatic activities were significantly reduced in a group of rats given 1.5% saline to drink for 30 days. The results were assumed to be caused by suppression of the renin-angiotensin system due to chronic salt ingestion.

In another group of rats that were administered injections (ip) of 5-hydroxytryptophan simultaneously with injections (sc) of estradiol benzoate, PRA and PCEA decreased by 100% and 131.2%, respectively, from that of the control animals. The serotonin hormone precursor, 5-HTP, apparently has a nephrotoxic effect on the kidney, thereby causing

decreased enzymatic activities.

The ability of the radioimmunoassay procedures used to detect very low concentrations of A I and A II was determined prior to the assay on experimental plasma samples. Plasma from 36 hr 2-kidney nephrectomized rats and plasma from a group of SHR's was assayed to make these determinations. PRA and PCEA were significantly decreased in plasma from the 2-kidney nephrectomized rats due to the removal of the source of the renal enzyme (renin). PRA was 60 times greater in the SHR's plasma than that observed in the 2-kidney nephrectomized rats. In the meantime PCEA was increased by approximately 700%. While these results show the validity and effectiveness of the radioimmunoassay, they simultaneously implicate the converting enzyme (CE) as an important factor in regulating the levels of A II in plasma which subsequently regulate blood pressure. These results further imply that it is important to measure converting enzyme activity (CEA) under conditions in which the renin-angiotensin system is suspected of playing a role. This is evident from these results since regulation of CEA might not be reflected in measured PRA. Under these conditions the importance of CEA is a better index of hypertension of a renal orgin than renin, because the action of the CE is the last step in the enzymatic reactions of the renin-angiotensin system, with the subsequent liberation of A II.

ACKNOWLEDGMENTS

I wish to express eternal thanks to my mother and father, who inspired in me the value of the necessity to obtain an education.

I am also grateful to my graduate advisor, Dr. Joseph B. Myers, for guidance and instruction throughout this period of study at Atlanta University.

Finally, but none in the least do I express gratitude and thanks to my wife, Grace, and daughter, Damita, for their patience and encouragement during the pursuit of this study.

This investigation was supported (in part) by the National Institutes of Health MARC Fellowships No. 5-F14-GM-56014 and 1-F32-GM-05566 from the National Institute of General Medical Sciences.

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CHAPTER I

INTRODUCTION

The renin-angiotensin system has been under investigation for many years. It has been established that the renal enzyme, renin, acts on the renin substrate, antiogensinogen. This reaction results in the formation of the decapeptide angiotensin I (A I), generally believed to be biologically inactive. To achieve biological activity, A I must be cleaved by a converting enzyme in the circulation. This reaction produces the biologically active octapeptide angiotensin II (A II). Based on these observations, the converting enzyme is the apparent cornerstone in the renin-angiotensin system, yet little is known about its physiological activity.

The discovery and description of the A I converting enzyme from plasma may be dated from the early work of Skeggs et al. (1954,1956) and that of Helmer (1957). Except for the work of these investigators, and especially Ng and Vane (1968), who indicated that most of the angiotensin conversion occurs in the lungs, little is known about the converting enzyme. Early studies with this enzyme implied that the primary site whereby A I was converted to the active form, A II, was localized in plasma. It was soon realized however, that the rate of conversion by the plasma enzyme was not sufficient to account for the rapid rise in blood pressure following intravenous injection. The tissue responsible for the primary conversion remained unknown until the work of Ng and Vane (1968). They found that the primary site of conversion appeared to be

the lungs. Support for this site was also reported by Huggins and Thampi (1968) who observed that under in vitro conditions, lungs contained a relatively high content of converting enzyme. Cushman and Cheung (1969) reported studies on the purification of A I converting enzyme from dog lung homogenates and described some of its kinetic properties. Oparil et al. (1970) have shown a rapid pulmonary conversion of tritium-labeled A I under in vivo conditions.

The first method for measuring plasma converting enzyme activity (PCEA) was reported by Skeggs et al. (1956) and was based on an eight-tube countercurrent distribution procedure for the separation of A I from A II. Helmer (1955) used a spirally cut strip of rabbit aorta which responded to A II but not to A I, in order to measure the capacity of human plasma to form A II. More recently, A I labeled at the C-terminal amino acid was used by Huggins and Thampi (1968) for the measurement of A I converting enzyme activity. Boucher et al. (1970) used a cation exchange adsorption technique for the same purpose. The procedure consisted of the elution of A I and II adsorbed on one column of 50W-X8 (NH_4^+) Dowex resin into a second column. A I was adsorbed on the second column of 50W-X8 Dowex resin, while A II went through and was collected in a siliconized flask and later lyophilized. The amount of A II formed by the action of the converting enzyme on the A II substrate after a 20 min incubation period at 47 C was measured by biological assay using nephrectomized rats. Reproducibility was within a range of 15%. All these methods essentially measure A II once it has been separated from A I, or they measure A II in the presence of A I on the assumption that the radioimmunoassays are highly specific for both angiotensins.

The development of radioimmunoassay techniques, with their application to low molecular weight peptides, has permitted the measurement of A I and A II (Waite, 1972b). In general, it appears that there has been little difficulty in raising antisera of high specificity and avidity to both A I and A II. These antibodies have been raised against immunogens prepared in a variety of ways. The immunogen complexes have consisted of angiotensin with porcine gamma globulin, rat serum albumin (Goodfriend et al., 1964), poly-L-lysine (Haber et al., 1965) and microparticles of carbon (Peart, 1969). These various immunogens have been employed in different animals, and with varied immunization schedules. The animal most often used has been the rabbit. With the presence of tyrosine in the compound, the preparation of labeled A I with ^{125}I or ^{131}I of high specific activity has been achieved with comparative ease using the chloramine-T method of Hunter and Greenwood (1962).

The development of suitable extraction procedures has in recent years permitted the measurement of the concentration of A II in blood or plasma. Until recently, however, the radioimmunoassay of A I has been limited to the measurement of plasma renin concentration. More recently, suitable techniques for the extraction of A I have been developed and the circulating levels of A I have been measured. Using the assay technique of Brown et al. (1966) to measure plasma renin concentration, and the technique of Dusterdieck and McElwee (1971) to measure plasma A II, Waite (1972a) found a significant relationship between plasma renin and plasma A II concentrations and demonstrated that there is a strong positive correlation between A I and A II.

Current enzyme assays are most often performed by allowing the

enzyme to act on its substrate under standard conditions with subsequent analysis of the product. This principle is also used when the product is measured by radioimmunoassay; the antibody against the product is added after the enzymatic incubation has taken place. Poulsen (1971) introduced a simplified method for the radioimmunoassay of enzyme systems. His procedure introduced a new application for radioimmunoassay. The principle consists of the presence of an excess of radioimmunoassay antibody at the start of the enzymatic incubation. The antibodies can capture an intermediate product such as angiotensin formed with time, and thereby, protect it against enzymatic degradation. Subsequent to the enzymatic reaction, the incubation mixture is diluted and labeled angiotensin is added simultaneously. Finally, free angiotensin is removed with charcoal, and counting of the antibody-bound angiotensin gives the amount of captured angiotensin with an accuracy of ±0.04. The method is simple and the whole procedure, except for counting, is performed in the same tube.

This assay, if used for A II, can be used with an analogous assay for A I in order to ascertain the activities of the converting enzyme. At present it is possible to obtain highly specific affinity antibodies against both hormones (A I and A II) with less than 5% cross-reactivity. Therefore, the major purpose of this investigation was to study the activity of the converting enzyme of the renin-angiotensin system during the development and maintenance of hypertension in one model of spontaneously hypertensive rats (SHR) and three models of experimentally induced hypertensive rats. The hypertensive models are as follows: 1) one kidney Goldblatts, 2) salt-induced, 3) 5-Hydroxy-DL-tryptophan

induced, and 4) SHR's. The antibody capture technique for A II proposed by Poulsen (1971) was used to elucidate these phenomena, with subsequent radioimmunoassay analysis of the A I and A II hormones in the plasma of each hypertensive model.

CHAPTER II

REVIEW OF LITERATURE

Various aspects of the renin-angiotensin system have been studied for many years. The first observations were made by Tigerstedt and Bergman in 1898, when they extracted renin from rabbit kidneys and showed that it would raise the blood pressure in dogs and that its activity could be destroyed by boiling. Little interest was taken in these observations until 1934 when Goldblatt and his colleagues showed that the application of a clip to one renal artery would raise the blood pressure in the experimental animal (Goldglatt et al., 1934). Consequently, for about 36 years after its discovery renin was only a curiosity and a matter of dispute because no particular attention was paid to the enzyme's possible significance since the idea of primary (non-renal) hypertension was dominant. According to Berman and Vertes (1973) it is no exaggeration to call Goldblatt's experiment one of the most important in modern science for it not only reestablished the forgotten enzyme, but also led to some of the modern concepts of hypertensive disorders.

After Goldblatt's experiment many investigators began to look again at the possibility of release of pressor substances from such a kidney, and renin was rediscovered in kidney extracts (Pickering and Prinzmetal, 1938). The subsequent work of Page and Helmer (1940) and Braun-Menedez et al, (1939,1940) led to the discovery that renin was an enzyme which acted on a substrate in the plasma to produce pressor activity called, initially, angiotonin by Page and his associates and hypertensin by

Braun-Menendez and his coworkers. The name of this pressor substance was later changed by agreement and called angiotensin (Page, 1975). It has subsequently been shown that this activity is due to the production of a decapeptide which is readily changed to an octapeptide in the circulation (Skeggs et al., 1955,1956; Elliott and Peart, 1956,1957).

Early efforts to isolate and characterize angiotensin were hampered by the presence of angiotensinase (peptidase) in renin and renin substrate preparations. The problem was overcome by using techniques which destroyed peptidase activity by acidification (Poulsen, 1969a) and by charcoal adsorption of angiotensin as soon as it was formed, thus rendering it inaccessible to peptidase activity or by preliminary purification to exclude angiotensinase from the system (Peart, 1955,1956; Boyd et al., 1969). First, horse angiotensin (Skeggs et al., 1955), then bovine (Elliott and Peart, 1956,1957), hog (Bumpus et al., 1957) and human (Arakawa et al., 1967) angiotensin I (A I) were isolated. By the use of several amino acid analysis techniques, both Skeggs et al. (1955) and Elliott and Peart (1956,1957) were able to concurrently identify the amino acid sequence in A I; the sequence was Asp.Arg.Val.Try.Ile.His.Pro.Phe.His.Leu, respectively. The first pure A I was obtained by Skeggs et al. (1954) from the incubation of pig renin with renin substrate obtained from horse plasma. The material was shown to be a decapeptide, with nine different amino acids and with an isoelectric point of 7.7. Its amino acid composition was substantiated by Lentz et al. (1956) when they demonstrated the amino acid composition of hypertensin II and showed its biochemical relationship to hypertensin I. Confirmation of the sequence has since been made through synthesis of the peptide by

Bumpus et al. (1957) and Rittel et al. (1957) during the period when the Merrifield solid-phase method of synthesis was unknown (Page, 1975). Human, horse and hog A I are identical in their amino acid sequence, but bovine A I differs in that valine replaces isoleucine in the five-carbon position.

Skeggs et al. (1954) discovered two forms of angiotensin that could be separated by a 50 tube countercurrent distribution method. One of the products (A I) was the decapeptide produced by the action of renin on the renin substrate (angiotensinogen). The second product (A II) was the octapeptide formed after the removal of the terminal histidyl-leucine from A I. In the absence of plasma, A I exhibited no significant vasoconstrictor properties, either on isolated rabbit aortic strip (Helmer, 1957) or the perfused isolated rat kidney (Ng and Vane, 1968) whereas, A II was highly active. Such observations led to further awareness of an enzyme in plasma that converts the decapeptide to an octapeptide.

The work of Page and Helmer (1940) and of Braun-Menendez and his colleagues (1940) demonstrated that renin was not, by itself, a pressor agent and that some factor in plasma was necessary to produce its pressor activity. Both groups, therefore, suspected the existence of an activator, co-factor, or a substrate in plasma. Work by Plent et al. (1943) demonstrated that this co-factor was the renin substrate and that it could be characterized as a protein contained largely in the $\alpha 2$-globulin fraction of plasma. Subsequently, in a series of important experiments, Skeggs et al. (1964) provided considerable information on the nature of renin substrate. This group was able to degrade horse renin substrate by treatment with trypsin and thereby obtain a 14-amino acid residue

polypeptide. This tetradecapeptide yielded A I upon incubation with renin. The first 10 amino acids from the N-terminal group were identical with those found in A I. The structure of the tetradecapeptide was later confirmed by synthesis and it was established that renin acts on this substrate at the leucyl-leucyl bond to liberate A I and a tetrapeptide. The organ source of the circulating renin substrate (angiotensinogen) has not been established unequivocally but a hepatic source seems likely. Observations by Page et al. (1941) and Leloir et al. (1942) indicate that hepatectomy reduces renin substrate. Studies by Drury et al. (1951) indicate that hepatectomy also eliminates responses to injected renin. In support of these studies are other observations indicating that plasma renin substrate may be very low or at times even undetectable in patients with liver disease (Ayers, 1967). Renin substrate has not been identified in extracts of liver, spleen, lungs or heart (Munoz et al., 1940). However, more subsequent studies indicate that hepatic tissue cultures can generate renin substrate.

The current state of knowledge of the components of the renin-angiotensin system wherein renin, after its release into the circulation, acts on its substrate (angiotensinogen) to produce the decapeptide (A I), and its subsequent conversion by the converting enzyme to the biologically active octapeptide (A II), is due to the work of Bumpus et al. (1961), Peart (1965) and Brown et al. (1966). According to Peart (1969) it is apparent that the amount of substrate present in the system affects the rate of reaction, and the rate of conversion of deca- to octapeptide will limit the amount of biologically active end product appearing in circulation. The work of Ng and Vane (1968) demonstrated that large

amounts of A I are converted to A II in transit during a single passage through the lungs rather than in the circulation itself. While in circulation A II is subject not only to the degradative action of the various angiotensinases present in blood, but also to clearance in transit through tissues. It is therefore likely that most of the metabolic clearance is carried out within the tissues rather than in the blood.

According to Vane (1969) renin has a long half-life in the circulation (15 min) in comparison with a single circulation time of 15 to 30 sec. It therefore circulates again and again, and the gradual decrease in its concentration is due to liver inactivation. By its action on angiotensinogen, renin generates A I especially on the venous side of circulation and it is relatively stable. However, A I is subsequently converted to the more active A II as it passes through the lungs. A I formed on the arterial side remains unconverted and most of it (50 to 70%) disappears in the peripheral vascular beds without being converted to A II (Ng and Vane, 1968). The 30 to 50% of A I which survives passage through peripheral vascular beds mixes with that which is converted to A II in the lungs. It is not known how quickly the angiotensinases in the blood degrade A I but any such degradation is probably slow.

A II formed in the lungs is stable in blood (half-life, 3 min) compared with its disappearance (50 - 60%) in a single passage through peripheral vascular beds. That which passes through the peripheral vascular beds recirculates, however, there is no removal of A II in pulmonary circulation (Hodge et al., 1967).

The renin-angiotensin system is evidently an enzyme system in which

renin is the enzyme and angiotensin the product of the enzymatic reactions (Skeggs et al., 1959). A II is responsible for all the known effects of the system. It is the most effective pressor substance and influences the salt and water balance through its effect on the kidneys and adrenals. The interest in the system is due to the unsolved question whether or not the renin-angiotensin system is active in the production of the various types of hypertension (Poulsen, 1970).

Renin is a proteolytic enzyme that is synthesized, stored and secreted mainly by the kidney. Oparil and Haber (1974) reported that renin-like enzymes have been extracted from a variety of organs, including the uterus, placenta, fetal membranes, amniotic fluid, brain adrenal glands, and the submixillary glands of the white mouse. Extrarenal sources have been used to explain the occasional finding of renin activity in the blood of anephric subjects (Capelli et al., 1968), but there is yet no evidence that these extrarenal sources have any physiologic role in blood pressure regulation or that the enzymes are identical with renal renin.

Although the enzyme is found in high concentrations in various organs, renin is concentrated mailny in the epithelial cells of the juxtaglomerular apparatus in the kidney (Fraup, 1968). The only known place in which all of the components of the renin-angiotensin system are found together is in plasma. Poulsen (1970) reported that the half-life of renin is 24 - 90 hr whereas that of the converting enzyme is in the range of 2 - 10 min, and the peptide enzymes (angiotensinases) approximately 5 min. Based on these data renin is the rate-limiting enzyme.

In spite of this, the concentration in plasma of the biologically active octapeptide A II, is not solely determined by the concentration of renin.

Observation of the actions of the angiotensins led to the study of the enzymes involved in the metabolism of the liberated peptides, including the A I converting enzyme. The A I converting enzyme was discovered by Skeggs and his associates in the mid 1950's when they noticed that horse plasma contains an enzyme that converts A I to A II (Skeggs et al., 1954,1956; Lentz et al., 1956). They reported that renin releases the decapeptide (A I) from angiotensinogen and that this decapeptide is in turn converted to the octapeptide (A II) when the converting enzyme cleaves a histdylleucine dipeptide from the C-terminal end of A I. The enzyme requires chloride ions and is inhibited by ethylenediamine tetraacetic acid (EDTA) or other chelating agents such as dimercaprol (BAL) or 8-hydroxyquinoline. These observations strongly suggest that the enzyme is a metalloenzyme. Around the same time Helmer (1955) also observed the existence of a factor in plasma that activated angiotensin preparations.

After these discoveries, the matter lay dormant for a long time, because A I was not available in pure or synthetic form in substantial quantities. The issue of conversion was kept alive, however, because of the difference between the effects of A I and A II on isolated smooth muscle preparations. It was observed that A I must be converted to A II before it becomes active in most biological systems in vitro (Skeggs et al., 1956). By means of a spirally cut strip of rabbit thoracic aorta Helmer (1957) demonstrated that angiotensin exists in two forms.

One form, A II, causes a contraction of the strip. The other, A I, is inactive. They are equally pressor when injected intravenously in animals. An enzyme in plasma converts the inactive form to the active form. The identical pressor activity can be explained by the excess of the converting enzyme in the plasma of the intact animal which rapidly converts A I to A II. Bumpus et al. (1961) used the isolated rat uterus for the same purpose. Anderson (1967) reported a similar bioassay technique. Several chromatographic procedures for separating A I and II have been described. A I labeled at the C-terminal amino acid was used by Huggins and Thampi (1968) for the measurement of A I converting enzyme in rat plasma, heart, brain, liver, diaphragm, kidney, lung, aorta and uterus.

Helmer (1957) also reported that some patients with hypertension have a greater content of the converting enzyme in their plasma than is found in normotensive subjects. It was suggested that in addition to the converting enzyme, other factors in plasma may enhance the ability of angiotensin (i.e., catecholamines, prostaglandins, norepinephrine, etc.) to induce constriction of tissues in bioassays. These factors may sensitize the mechanisms in the muscle tissue which set up the process of contraction.

Erdos (1975) reported in a review article that the need for such conversion in vivo is not that noticeable because the two peptides have similar effects on systematic blood pressure after intravenous injection due to the rapid conversion of A I in the body. It has been difficult to measure the conversion of A I to A II by bioassay, because tissues which contain the converting enzyme also inactivate the released peptide. Estimation of conversion in vitro gave only semiquantitative data (Bumpus et al., 1961) until radioactive substrates became available.

The measurement of renin activity, concentration and substrate has been widely performed by bioassay of the A I generated during incubation of plasma samples in vitro (Boucher et al., 1964; Pickens et al., 1965; Helmer and Judson, 1963). Recent studies in human beings (Skinner et al., 1969) and rats (Menard et al., 1970) have demonstrated that measurements of all three parameters concerned with renin activity in vivo—plasma renin activity (PRA), plasma renin concentration (PRC), and plasma renin substrate (PRS)—are necessary for complete evaluation of changes in the renin-angiotensin system. In small animals such as the rat, simultaneous measurement of these parameters has not been possible due to the volume of plasma required for use with the bioassay (Menard and Catt, 1972).

Radioimmunoassays for A I have been applied to the measurement of PRA in man, giving results which appear to correlate well with those obtained by bioassay (Boyd et al., 1969; Haber et al., 1969; Waite et al., 1972a). As radioimmunoassay of A I is considerably more sensitive than bioassay methods, Menard and Catt (1972) applied it to the assay of PRA, PRC, and PRS during studies in the rat, and developed methods which allow all three measurements to be performed on small blood samples. These methods are applicable to the assay of renin parameters in the plasma of other species. They also allow for the simultaneous measurement of all three parameters in the renin-angiontensin system.

Regardless of the method used in many situations, Boyd et al. (1967, 1969) concluded that the bioassay methods available for the estimation of A II in human plasma did not possess sufficient sensitivity for this hormone in various physiological and pathological states, therefore,

they considered the use of radioimmunoassay techniques. A major difficulty with attempts to apply the radioimmunoassay technique to A II had been the problem of developing a suitable antibody. A II was found to be an octapeptide with a small molecular weight of approximately 1,000 and antigenically weak. Some success had previously been achieved by immunizing with angiotensin which had been covalently linked to larger carrier molecules (Haber et al., 1965; Catt et al., 1967). Recently, radioimmunoassays for angiotensin III (A III), based on such antibodies, have been reported from two separate laboratories (Vallotton et al., 1967; Catt et al., 1967).

In an effort to achieve maximum avidity and specificity of the antibody to A II, Boyd et al. (1969) reported success in their attempts to raise antibodies only against the free angiotensin molecule itself. These investigators raised antibodies in rabbits by immunizing with Val^{15}-A II amide adsorbed onto micro-particles of carbon. According to the method of Peart (1955), carbon was used as the adsorbent because of its high affinity for A II and its ability to protect the molecule against the action of the plasma angiotensinases. The subsequent use of such high titre antibodies in the radioimmunoassay of A II in human circulating plasma has been reported (Boyd et al., 1967, 1969). The test used in these experiments was able to detect 30 pg amounts of A II and was not influenced significantly by A I.

Since 1967 other investigators (Vallotton et al., 1967; Catt et al., 1967; Sundsfjord, 1970; Dusterdieck et al., 1971; McBride et al., 1971) described radioimmunoassays for A II which detect small amounts of the peptide, but lengthy extraction and concentration procedures were

required. Reports by Vallotton et al. (1967) and Goodfriend et al. (1968) also indicated a lack of agreement between measurements of A II (generated in vitro by incubation with renin) obtained by the rat pressor assay and that measured by radioimmunoassay of the same sample.

Gocke et al. (1968) described the range of concentration of A II in normal human subjects and documented the variations in A II concentrations with changes in sodium intake. These workers also correlated the variations with renin activity. Their findings were obtained using a simple rapid radioimmunoassay procedure capable of measuring A II directly from 0.1 ml of human plasma. The validity of the measurement was substantiated by the ability of the immunoassay to duplicate results obtained by the pressor bioassay of the A II generated in vitro by incubation with either endogenous or added renin. In experiments performed by Gocke et al. (1968), A II was generated by incubation at 37 C with endogenous renin for 24 hr or with increments of highly purified human renin for 30 min. Excellent agreement between bioassay and immunoassay determinations was obtained except when 0.003 M EDTA was present in the renin-generation step. In this case, less than 1% of the A II found by bioassay was detected by the radioimmunoassay. This was most likely caused by the inhibition of the conversion of A I to A II by the presence of EDTA. In most radioimmunoassay procedures reported, the antibody shows very little cross-reaction with the decapeptide. Thus, the antibody fails to recognize the A I formed. However, on injection of the sample into the rat for bioassay, A I is rapidly and quantitatively converted to the pressor substance, A II. When the plasma was dialyzed to remove the EDTA prior to the incubation step full agreement between the two assay methods was obtained. This correlation of immunoassay

and bioassay measurements provided important verification that both methods measure the same substance and ruled out the possibility that degradation products or other factors in plasma cross-react with the antibody to any significant extent.

Sensitive and reproducible bioassays have been used to elucidate much of what is known of the renin-angiotensin system. Most of the procedures were based on the ability of A II to cause smooth muscle contraction. Biologic response was assayed either directly or after an incubation step in which the renin-substrate reaction was allowed to take place under standardized conditions, thus generating angiotensin and giving a measure of renin activity. The biologic response was either contraction of rabbit aorta (Helmer, 1957) or rat colon (Needleman et al., 1972) or the systematic pressor effect in an intact nephrectomized rat (Boucher et al., 1961). Responses were related to control injections of standard A II. Although precise and reproducible measurements of plasma renin activity have been obtained by bioassay in many research laboratories, the wide clinical application of this approach has been limited by inconsistent standardization and reproducibility, undue time requirement for sample processing, and frequently a lack of sensitivity and specificity (Oparil and Haber, 1974).

Radioimmunoassay for A II and A I became possible because highly purified synthetic peptides for use as antigens became available. By coupling angiotensin to a macromolecular carrier such as poly-L-lysine (Haber et al., 1965), a protein (Deodhar, 1960; Goodfriend et al., 1964) or finely divided charcoal (Boyd and Peart, 1968) an effective immunogen was created. The hapten-carrier complex gave rise to specific high affinity antibodies. Studies with peptide analogues and degradation products of A I and II showed that there was less than 5% cross-reactivity

between most anti- A I and anti- A II antibodies (Haber et al., 1965). Furthermore, except for the carboxyl hepta-peptides and hexa-peptides, the degradation products of A II were nonreactive with most anti-angiotensin antibodies (Dietrich, 1967). Vallotton (1971) reported that since the structural requirements for biologic and immunologic activity in the A II molecule are similar, fragments of A II do not impair to any great degree the usefulness of the A II radioimmunoassay in circulating plasma.

Labeled peptides of high specific activity for the radioimmunoassay procedures were obtained with ^{125}I by the chloramine-T technique of Hunter and Greenwood (1962). A modification of the procedures made it possible for the production of a monoiodinated peptide by Nielsen et al. (1971) that is stable for many months. Separation of bound from free antigen is generally done by a modification of the charcoal method of Herbert et al. (1965), or by the antibody-capture technique of Poulsen (1971). Radioimmunoassay procedures for A II were developed before those for A I because of the earlier availability of the octapeptide as an antigen. The A II assays are sensitive enough to measure circulating levels of A II in normal man (Boyd et al., 1967; Catt et al., 1967; Gocke et al., 1968; Goodfriend et al., 1968; Page et al., 1969; Sundsfjord, 1970). Oparil and Haber (1974) reported that the apparent discrepancies between methods can be attributed to differences in sodium intake and posture of the subjects and a variability in extraction techniques. According to Dietrich (1967) and Haber et al. (1969) levels of A II correlate well with renin activity.

The use of the A II assay to determine renin activity has been less

successful. When an assay for A II is used to measure renin activity, in vitro conversion is necessary. Conditions favorable for the action of plasma converting enzyme also favor angiotensinase action causing simultaneous generation and destruction of A II (Oparil and Haber, 1974). Use of a specific anti- A II antibody eliminates the conversion step and permits a more direct accurate measurement of renin activity (Haber et al., 1969; Boyd et al., 1969; Hollemans et al., 1969; Lehfeldt and Hutchens, 1971; Cohen et al., 1971). The inhibitors of converting enzymes that are added to the incubation mixture also inhibit angiotensinase activity, ensuing improved recovery of the generated peptide.

All of the commonly used radioimmunoassay procedures for renin activity employ an initial incubation step in which endogenous renin and substrate react to generate A I. The reaction is then stopped usually by freezing. ^{125}I labeled A I and specific anti- A I antibody are added at a later time and allowed to equilibrate with the generated A I in a second incubation step. Bound and free forms of ^{125}I - A I are then separated by conventional techniques and quantitated by gamma or scintillation counting. The amount of A I present is determined by a standard curve, according to the methods of Bernson and Yallow (1968). Oparil and Haber (1974) maintained that there are three main areas in which variations in procedure have been introduced: pH of the initial incubation, choice of enzyme inhibitors, and duration of the initial incubation. Detailed evaluations and comparisons of methods for measuring renin activity are currently being carried out in a number of laboratories. It is hoped that the standardization of renin and angiotensin preparations, incubation conditions for generating angiotensin, and units of expressing renin activity will facilitate comparison of renin activity measurement from laboratory

to laboratory (Oparil and Haber, 1974).

Variability in incubation conditions for generating angiotensin and in angiotensin standards has made comparison between bioassay and radioimmunoassay of plasma renin activity difficult to interpret. In one study comparing normal values obtained in a number of laboratories, values for renin concentration obtained by radioimmunoassay in reference to an international renin standard were lower than bioassay values (Haas and Goldblatt, 1972). With the use of the same plasma extracts for assay, A I concentration has been reported to be consistently lower by radioimmunoassay than by bioassay (Cohen et al., 1971; Menard and Catt, 1972), or comparable to bioassay values (Kotchen et al., 1973). Oparil and Haber (1974) suggested that the resolution will require measurement of angiotensin samples generated under identical conditions by both bioassay and radioimmunoassay with the same angiotensin standard in both procedures.

It is difficult to measure the concentration of circulating A I with accuracy. In vitro reaction of renin with its substrate, particularly prominent in plasma that contains large amounts of renin or has been allowed to become warm, cause false elevation of A I levels in unincubated samples. Some antisera react with plasma components other than A I and give falsely high results with unincubated plasmas (Page et al., 1971). Appropriate selection of antisera will provide for the elimination of this problem, since not all antibodies manifest such nonspecific cross-reactivity (Oparil and Haber, 1974).

CHAPTER III

MATERIALS AND METHODS

Materials

Male Sprague-Dawley and Wistar spontaneously hypertensive rats (SHR's) used in this investigation were obtained from Charles River Breeding Laboratories, New York, N. Y. Systolic blood pressures of all animals were monitored using a Programmed Electro-Sphygmomanometer (model PE-300). This instrument was connected to a desk model Physiograph recorder (model DMP-48), from which readings were made. Both instruments were purchased from Narco Bio-Systems, Inc., (Houston, Texas).

The following chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.); β-estradiol-3-benzoate, 5-hydroxy-DL-tryptophan, neomycin sulfate, bacitracin, Tris (hydroxymethyl) aminomethane (Trizma base), Tris (hydroxymethyl) aminomethane hydrochloride (Trizma HCl), ethylenediamine tetraacetic acid (EDTA), diidopropylfuorophosphate (DFP) and bovine serum albumin (BSA).

Materials used in the radioimmunoassay procedures for the measurement of angiotensin I (A I) and angiotensin II (A II) were purchased from New England Nuclear Biomedical Assay Laboratories (Worcester, Mass.); A I (5-L Isoleucine) [tyrosyl-^{125}I], A II (5-L-valine)[tyrosyl-^{125}I] amide, A I antiserum, A II antiserum, maleate buffer concentrate, A I standards, 8-hydroxyquinoline sulfate, dimercaperol (BAL) and Riafluor (liquid scintillator).

Radioimmunoassay grade charcoal and Dextran T-70 were purchased from Schwartz/Mann (Orangeburg, New York). Synthetic human A I and II were obtained from Beckman Bioproducts, (Palo Alto, Cal.). Sodium pentabarbital (Nembutal, 60 mg/ml) used for anesthetization was purchased from Mosher, Inc., (Atlanta, Ga.). All reagents and stock solutions were prepared in distilled or deionized water.

Gamma emissions from radioactive (monoiodinated) A I and II samples were counted in a Beckman Liquid Scintillation Counter (model LS-230, Beckman Instruments, Inc., Atlanta, Ga.). Centrifugations were done in a Lourdes refrigerated centrifuge.

Methods of Procedure

All animals were fed Purina rat chow and tap water ad libitum unless specified otherwise. Approximately 125 rats (initial avg. wt. 210-250 g) were divided into 2 groups of controls, 1 group of doubly nephrectomized rats, 3 experimental (induced) hypertensive groups and 1 group of spontaneously hypertensive rats.

Blood pressure measurement

Systolic blood pressures of all animals were monitored 0-24 hr prior to any experimental procedure. The tail cuff blood pressure method was used, wherein a pneumatic cuff was placed around the base of the tail. A pneumatic bulb connected to a pulse transducer was then secured (taped) over the caudal artery. In this system, the animals were enclosed in a warming chamber in order to increase blood circulation which usually resulted in good pulsatile waves in the caudal artery. At all times during the monitoring of blood pressures, the animals were secured in specially built rat restrainers in order to minimize movement. A

systolic blood pressure above 150 mm Hg in unanesthetized rats was regarded as hypertensive (O'Steen et al., 1969).

Surgical procedures

All procedures involving surgery were performed after anesthetization with sodium pentabarbital (Nembutal, 40 mg/kg, ip). Operations were done under aseptic conditions. Surgery involved opening of the peritoneal cavity by making a mid-line incision along the linea alba (2.0 - 2.5 cm in length). These procedures involved either placing a clip around the left renal artery and removal of the right kidney (1-kidney Goldblatt), removal of the right kidney leaving the left kidney intact (1-kidney nephrectomized), or removal of both kidneys (2-kidney nephrectomy). After surgery the abdominal cavity was closed by suturing with size 0-4 silk Ethicon surgical suture. The outer incision was then closed with metal wound clips.

Collection of blood samples

Blood samples were collected at 10,20 and 30-day intervals after each rat was decapitated. The blood was allowed to flow into 200 ml beakers that had been rinsed with a 0.3% sodium citrate solution and placed in an ice water bath (0-4 C). The samples were then measured and poured into 10 ml polypropylene centrifuge tubes containing sodium citrate (final concentration, 0.3%). Subsequent to collection, the samples were centrifuged at 1,500 rpm in a refrigerated centrifuge (0-4 C). Plasma samples were then decanted and stored in 7 ml siliconized vacutainers at -20 C until assayed for A I and A II concentrations.

One kidney Goldblatt hypertensive model

Forty-five male Sprague-Dawley rats were weighed, anesthetized and

the right kidney removed. The left renal artery at the site where it joins the dorsal aorta was dissected out of its tissue bed and a clip (0.20-0.25 mm, i. d.) placed around the exposed artery. The clip was a modification of that used by Goldblatt et al. (1934) made from silver wire. The internal diameter of the clip varied according to the body weight of the animals. The 0.20 mm clip was used for rats weighing up to 200 g (initial avg. wt.), and for those weighing above 200 g a 0.25 mm clip was used. Controls for this model were 21 rats that had the right kidney removed and the left kidney intact. Nine experimental and 7 control rats were sacrificed at each designated time interval.

Salt-induced hypertensive model

The right kidney of 27 rats for this model was removed with the left kidney left intact. After a 3-day recovery period, all animals in this group were given 1.5% saline to drink. Controls consisted of 21 rats that had the right kidney removed, but given tap water to drink. Nine salt-loaded and 7 control rats were sacrificed at each 10, 20, and 30-day interval, and plasma samples collected.

Malignant hypertensive model

Fifteen rats were administered an intraperitoneal (ip) injection of 5-Hydroxy-DL-Tryptophan (50 mg/kg) dissolved in 0.9% saline and a subcutaneous (sc) injection of β-estradiol-3-benzoate in sesame oil (40 μg/0.1 ml) simultaneously. Controls for this group were given a sham injection of 0.9% saline (ip) and 0.1 ml sesame oil (sc). Injections were made on day 1, 7, 14, 21, 42 and 47 with all surviving animals being sacrificed on day 50 and plasma samples collected. Blood pressures of experimental and control animals were recorded at each time interval prior to injections

and on the day the experiment was terminated.

Spontaneously hypertensive model

The spontaneously hypertensive Wistar rats were direct descendants of the Wistar strain developed by Okamoto and Aoki (1963). Twelve of these rats (224-333 g) were sacrificed and plasma samples collected (approx. age, 28-38 wks.).

Radioimmunoassay of antiotensin I

The radioimmunoassay procedures used for the determination of A I concentrations in all plasma samples were based on protocols published by New England Nuclear Biomedical Assay Laboratories. The assay system is an adaptation of the radioimmunoassay methods of Haber et al. (1965,1969) and Goodfriend et al. (1968). The assay consisted of 0.1 ml aliquot each of a generated, and an endogenous sample of plasma. These samples consisted of 1.0 ml of plasma, 2.0 ml of 0.2 M maleate buffer (pH 6.0), and 0.1 ml of an inhibitor of both the plasma converting enzyme and the angiotensinases. The inhibitory mixture contained 5.0 mM EDTA, 0.32 M 8-hydroxyquinoline sulfate, 0.05 M DFP and 0.2% bactracin in distilled water. After vortexing, 1.0 ml of each plasma sample was incubated in a water bath at 37 C for 1 hr, the rest of the sample was left at 0-4 C in an ice water bath. To 0.1 ml of each of these plasma samples were added: rabbit A I antiserum in tris-acetate buffer (0.1 M, pH 7.4, 0.5 ml) and ^{125}I tyrosyl labeled 5-L isoleucine A I (approx. 8 pg/0.1 ml, specific activity 500-1000 μCi/mg, 5-6000 counts/min), 0.1 ml. The final volume of all samples was 0.7 ml and each sample was assayed in duplicate at 0-4 C.

Endogenous A I plasma samples were kept at 0-4 C at all times during

the assay, while samples of generated A I were incubated at 37 C for 1 hr. Generation of the 37 C samples were terminated by immersion of the sample into an ice water bath at 0-4 C. After an 18-24 hr incubation period of all samples at 0-4 C, free and bound A I were separated using a dextran T-70 treated Norit A charcoal suspension (pH 7.4). A total volume of 1.0 ml of the charcoal suspension was added to each plasma sample and the antibody bound and free A I separated by centrifugation for 20 min at 4 C (5,000 rpm). The term endogenous refers to the actual amount of A I in a plasma sample, while the term generated refers to the amount of A I measured after incubation in a water bath at 37 C for a period of 1 hr. All samples were assayed in 3.0 ml polystyrene test tubes (silicone treated).

After charcoal filtration of free from antibody bound A I, gamma emmissions of the supernatant were counted in a well type liquid scintillation counter. The process involved the use of 10.0 ml of Raiflour in 15 ml scintillation vials into which the plasma samples were added. Standards consisted of 0.1, 0.25, 0.5, 1.0, 2.5, and 5.0 ng/ml of lyophilized synthetic human A I (0.1 ml aliquots). Standards were assayed in the same manner as endogenous and generated plasma samples.

Procedures for calculating the values for each set of duplicate standards and the unknown A I concentrations of 37 and 4 C plasma samples were those suggested in the protocols for radioimmunoassay of A I published by New England Nuclear Biomedical Assay Laboratories. The net counts per min are expressed as a percentage of the total count value by use of the following equation:

$$\% \text{ Bound} = \frac{\text{average net counts}}{\text{average net total counts}} \times 100$$

Using semilogarithmic graph paper, the percent bound for each standard was then plotted against the amount of A I, as ng of A I added in the 0.1 ml standard solutions. The percent bound for the zero standard was about 50-60%. Determination of the amount of A I in ng for each plasma aliquot assayed was by interpolation from the standard curve. This was done by converting the ng of A I for both 4 and 37 C samples into ng/ml/hr by multiplying by 30 ($\frac{\text{ng X 3.0 ml}}{\text{0.1 ml}} = 30$). Plasma renin activity was expressed as ng/ml/hr, calculated by subtracting the 4 C value from the 37 C value (plasma renin activity = ng/ml/hr 37 C - ng/ml/hr 4 C).

Radioimmunoassay of angiotensin II

A modification of the antibody capture technique for A II proposed by Poulsen (1971) and assay procedures for A II developed in the Atlanta University laboratory were used for the determination of all plasma A II concentrations. A II standards (0.0025-2.0 ng per 0.1 ml) were made with synthetic human Asp^1, Val^5, A II (lyophilized) dissolved in 0.1 M tris-acetate buffer with 0.2% BSA and 0.1% sodium azide (pH 7.4).

The assay was performed in disposable 3.0 ml capacity polystyrene test tubes. All standards, and 4 and 37 C plasma samples were assayed in duplicate. The assay consisted of 1.0 ml of each plasma sample, 0.1 ml of 3 M tris-HCl (10 vol percent, pH 7.3) and 0.03 ml of neomycin sulfate (3 vol percent, 60 ng/ml) dissolved in 0.2 M tris-HCl, pH 7.5. After vortexing , 0.1 ml aliquots of each plasma sample was added to 0.1 ml of rabbit A II antiserum. Two tubes of each sample containing this mixture

were then incubated in a water bath at 37 C for 6 hr, while 2 tubes each were left at 0-4 C in an ice water bath according to the method of Poulsen (1971). Subsequent to the 37 C incubation period, an essential dilution was performed by the addition of 0.1 ml of A II (5-L-Valine)(tyrosyl-^{125}I) amide (approx. 4 pg/0.1 ml, specific activity 1000-1500 μCi/mg, 9-10,000 counts/min) and 0.7 ml of 0.1 M tris-acetate buffer (pH 7.4) to each 4 and 37 C tube (see table I for A II assay protocol). The final volume of all tubes was 1.0 ml. All samples were then incubated at 0-4 C for 18-24 hr. After the incubation period, free A II was separated from the bound A II using 1.0 ml of a 4% Norit A charcoal and 0.25% dextran T-70 suspension, with subsequent liquid A I.

To determine the concentration of A II in ng, each plasma aliquot assayed was interpolated from the standard curve after liquid scintillation counting. The curve was obtained from A II standards (.0025-2.0 ng per 0.1 ml) assayed in duplicate simultaneously with plasma samples (Table 1). The average net counts/min for all samples, total counts of controls and standards were obtained by subtracting from each the average blank counts/min. Percent bound was determined as in the radioimmunoassay for A I.

The ng of A II captured by the antibody for both 4 and 37 C samples were converted to ng/ml/6 hr by multiplying by 11.3 ($\frac{\text{ng X 1.13 ml}}{\text{0.1 ml}}$ = 11.3). Converting enzyme activity was expressed as ng/ml/6 hr of captured A II. This activity was obtained by subtracting the 4 C value from the 37 C value.

Table 1. Radioimmunoassay protocol for angiotensin II (A II) determinations.

Tube description	Buffer	5% BSA	Sample	Std.	Tracer	Ab	Charcoal
Total count	1.9	-	-	-	0.1	-	-
Blank	0.8	0.1	-	-	0.1	-	1.0
0 Standard	0.7	0.1	-	-	0.1	0.1	1.0
Standards	0.6	0.1	-	0.1	0.1	0.1	1.0
Samples	0.7	-	0.1	-	0.1	0.1	1.0

All concentrations are in ml.

Std. = Standard

Ab. = Antibody (antiserum)

CHAPTER IV

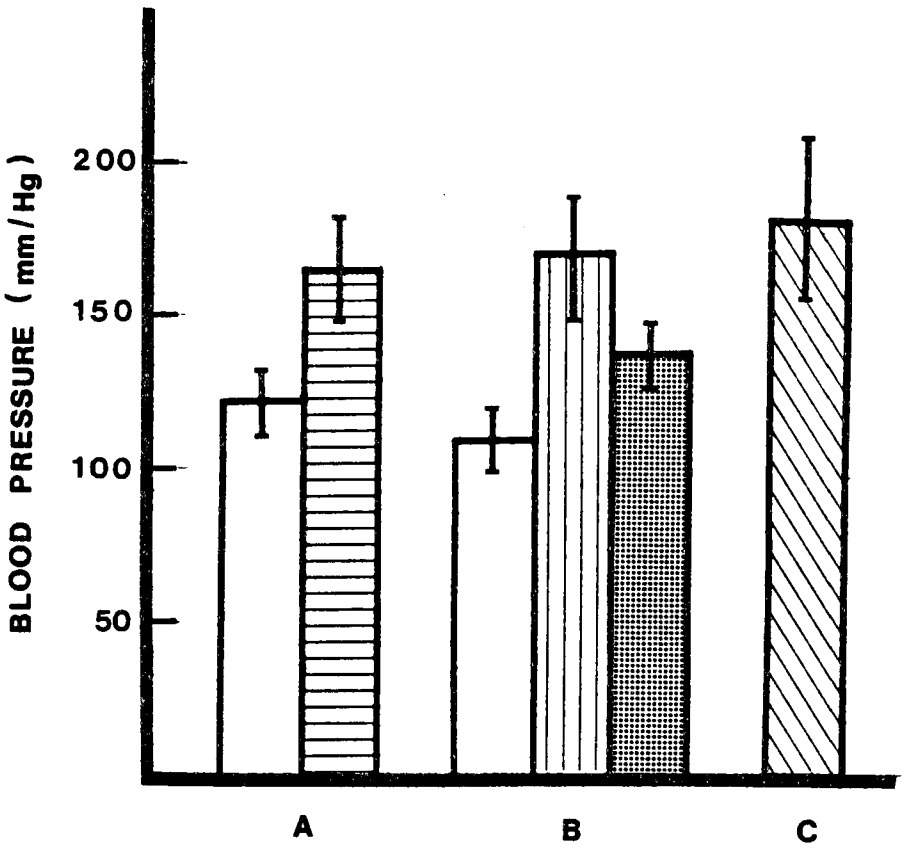
RESULTS

All data in the tables and figures are mean values with standard deviations (SD) of the mean. Statistical analyses were done according to the student t-test. The null hypothesis was rejected at the 0.05 (5%) level of probability. Computation of all data was performed at the Atlanta University Center Computation Facility.

Blood pressure measurements

The production of persistent hypertension in laboratory animals has provided methods for the investigation of various aspects of the renin-angiotensin system. Mean systolic blood pressures of 3 models of experimentally induced hypertensive rats produced for this purpose are presented in Figs. 1 and 2, and Tables 2, 3, and 4. In 3 groups of rats representing the one-kidney Goldblatt (1-K-G) model, terminal mean elevated blood pressures of 128.9, 141.8 and 169.3 mm Hg, respectively, were attained at the end of 10, 20, and 30-day periods (Fig. 2, Table 2).

Figure 2 and Table 3 show that terminal mean blood pressures of 127.6, 123.0 and 135.4 mm Hg, respectively, were observed in 3 groups of rats that were given 1.5% saline as drinking water for the same time periods after removal of the right kidney (salt-induced hypertensive model). Three groups of controls for both the salt-induced and 1-K-G models had terminal mean blood pressures of 108.6, 110.9 and 106.2 mm Hg at the end of the experimental periods. The experimental results show an elevation of blood pressure to approximately 164 mm Hg in the malignant hypertensive (5-hydroxy-DL-tryptophan, β-estradiol-3-benzoate injected) rats. The


BLOOD PRESSURE (mm/Hg)
200
150
100
50
A
B
C

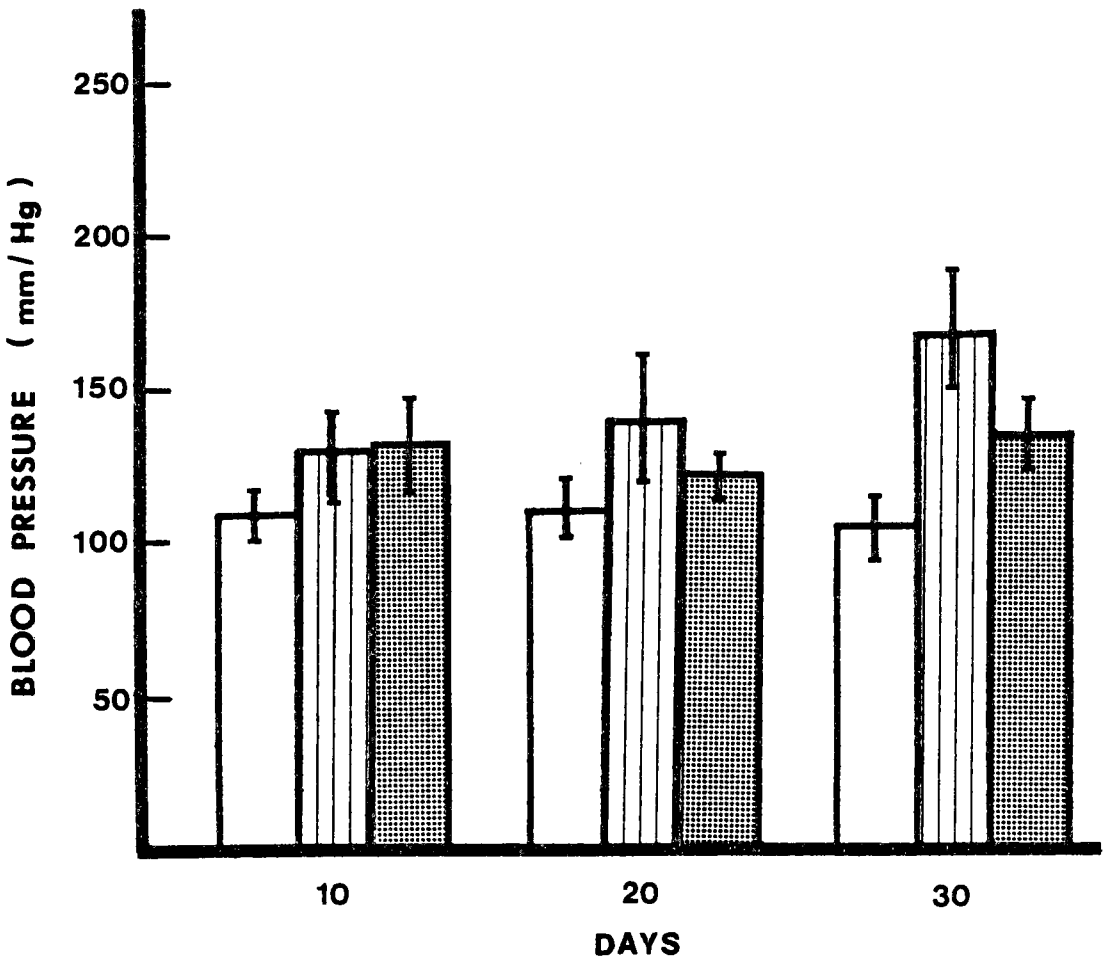

BLOOD PRESSURE (mm/ Hg)
250
200
150
100
50
10
20
30
DAYS

Table 2. Statistical data comparing one kidney Goldblatt (1-K-G) model to their controls, and values from 36 hr two kidney nephrectomized (2-K-N) and spontaneously hypertensive rats (SHR's).

Groups	(N)	Avg. Wt (g)	Mean B.P. (mm Hg)	Generated A I (ng/ml)	Endogenous A I (ng/ml)	PRA (ng/ml/hr)	Generated A II (ng/ml)	Endogenous A II (ng/ml)	PCEA (ng/ml/6 hr)
10 day controls	7	332.6	108.6 ±7.8	57.77 ±13.02	22.07 ±1.95	35.69 ±12.56	1.86 ±0.54	1.18 ±0.32	0.68 ±0.34
10 day Goldblatts	9	254.4	128.9 ±15.71	39.22 ±8.57	6.24 ±1.58	32.98 ±7.34	1.06 ±0.28	*0.61 ±0.12	0.45 ±0.19
20 day controls	7	385.3	110.9 ±10.76	31.67 ±7.98	6.19 ±2.78	25.47 ±6.06	1.06 ±0.42	0.70 ±0.25	0.36 ±0.20
20 day Goldblatts	9	331.7	141.8 ±20.21	*42.43 ±16.56	8.13 ±2.94	*34.29 ±14.12	1.13 ±0.34	0.65 ±0.17	0.47 ±0.23
30 day controls	7	427.7	106.2 ±8.28	33.42 ±12.65	6.44 ±2.33	26.98 ±11.30	0.93 ±0.28	0.61 ±0.22	0.31 ±0.17
30 day Goldblatts	9	419.4	*169.3 ±18.43	*59.74 ±44.65	*13.34 ±11.77	*46.40 ±33.54	*2.07 ±1.58	*1.33 ±1.23	*0.74 ±0.42
36 hr 2-K-N	5	300.6		3.98 ±0.98	3.52 ±0.86	0.45 ±0.25	0.56 ±0.12	0.49 ±0.10	0.06 ±0.06
SHR's	13	272.8	180.8 26.93	34.39 ±7.44	7.64 ±1.02	27.08 ±7.40	1.20 ±0.37	0.71 ±0.19	0.48 ±0.28

B. P. = Blood Pressure

PRA = Plasma renin activity.

PCEA = Plasma converting enzyme activity.

± = Standard deviation of the mean.

* = $P < 0.05$ (significant value).

All values represent the mean for the number (N) of rats assayed at each timed interval.

Table 3. Statistical data comparing salt-induced model to their controls, and values from 36 hr two kidney nephrectomized (2-K-N) and spontaneously hypertensive rats (SHR's).

Groups	(N)	Avg. Wt (g)	Mean B.P. (mm Hg)	Generated A I (ng/ml)	Endogenous A I (ng/ml)	PRA (ng/ml/hr)	Generated A II (ng/ml)	Endogenous A II (ng/ml)	PCEA (ng/ml/6 hr)
10 day controls	7	332.6	108.6 ±7.8	57.77 ±13.02	22.07 ±1.95	35.69 ±12.56	1.86 ±0.54	1.18 ±0.32	0.68 ±0.34
10 day salt-induced	9	268.0	127.6 ±15.54	*8.30 ±3.24	5.92 ±1.29	*2.38 ±1.47	*0.38 ±0.08	*0.32 ±0.08	*0.06 ±0.06
20 day controls	7	385.3	110.9 ±10.76	31.67 ±7.98	6.19 ±2.78	25.47 ±6.06	1.06 ±0.42	0.70 ±0.25	0.36 ±0.20
20 day salt-induced	8	270.9	123.0 ±5.55	*5.27 ±1.90	*3.76 ±0.39	*1.51 ±1.42	*0.37 ±0.07	*0.30 ±0.07	*0.06 ±0.04
30 day controls	7	427.7	106.2 ±8.28	33.42 ±12.65	6.44 ±2.33	26.98 ±11.30	0.93 ±0.28	0.61 ±0.22	0.31 ±0.17
30 day salt-induced	7	284.3	135.4 ±9.64	6.68 ±6.37	4.42 ±2.95	*2.26 ±1.61	0.54 ±0.32	0.36 ±0.12	0.18 ±0.11
36 hr 2-K-N	5	300.6		3.98 ±0.98	3.52 ±0.86	0.45 ±0.25	0.56 ±0.12	0.49 ±0.10	0.06 ±0.06
SHR's	13	272.8	180.8 ±26.93	34.39 ±7.44	7.64 ±1.02	27.08 ±7.40	1.20 ±0.37	0.71 ±0.19	0.48 ±0.20

B. P. = Blood pressure.

PRA = Plasma renin activity.

PCEA = Plasma converting enzyme activity.

± = Standard deviation of the mean.

* = $P < 0.05$ (significant value).

All values represent the mean for the number (N) of rats assayed at each timed interval.

Table 4. Statistical data comparing 5-hydroxy-tryptophan (5-HTP) model to their controls, and values from 36 hr two-kidney nephrectomized (2-K-N) and spontaneously hypertensive rats (SHR's).

Groups	(N)	Avg. Wt (g)	Mean B.P. (mm Hg)	Generated A I (ng/ml)	Endogenous A I (ng/ml)	PRA (ng/ml/hr)	Generated A II (ng/ml)	Endogenous A II (ng/ml)	PCEA (ng/ml/6 hr)
5-HTP controls	5	382.4	120.8 ±9.95	49.08 ±24.84	18.41 ±3.81	30.65 ±21.68	0.79 ±0.42	0.42 ±0.14	0.37 ±0.28
5-HTP experimentals	13	375.2	164.0 ±16.81	30.47 ±16.07	15.13 ±2.91	15.33 ±13.75	0.44 ±0.23	0.28 ±0.11	0.16 ±0.16
36 hr 2-K-N	5	300.6		3.98 ±0.98	3.52 ±0.86	0.45 ±0.25	0.56 ±0.12	0.49 ±0.10	0.06 ±0.06
SHR's	13	272.8	180.0 ±26.93	34.39 ±7.44	7.64 ±1.02	27.08 ±7.40	1.20 ±0.37	0.71 ±0.19	0.48 ±0.20

B.P. = Blood pressure.

PRA = Plasma renin activity.

PCEA = Plasma converting enzyme activity.

± = Standard deviation of the mean.

All values represent the mean of the number (N) of rats assayed at each timed interval.

controls for this group had a maximum mean blood pressure of 120.8 mm Hg (Fig. 1 and Table 4).

Terminal blood pressures for the 36 hr 2-kidney nephrectomized (2-K-N) rats were not measured. After nephrectomy, pulsations in the caudal artery only showed heartbeats per min, which could not be converted to blood pressure measurements. However, blood pressures of totally nephrectomized rats can be measured by tail plethysmography, but not by the tail cuff method used in our laboratory. When blood pressures were measured in these rats prior to removal of both kidneys, a mean blood pressure of 94.4 mm Hg was observed.

Mean blood pressure for the group of 13 SHR's used in this investigation was 180 mm Hg (Fig. 1). The terminal mean weight of all rats used for experimental purposes are presented in Tables 2, 3, and 4.

Validity of radioimmunoassay for determination of A I and A II concentrations

The following observations show the sensitivity or ability of the radioimmunoassay procedures used in this study to detect very low and high concentrations of A I and A II in plasma. All concentrations derived from such analysis of plasma samples are expressed as a mean value of the total number of animals in each experimental group. Table 4 and Fig. 3 show the resulting data computed after radioimmunoassay determination of the generated and endogenous concentrations of A II in the plasma of 5 rats that had been deprived of their source of renin (2-K-N) for 36 hr. These data, referred to as plasma converting enzyme activity (PCEA), are derived from the generated concentrations of A II minus the endogenous concentrations. The results reveal a PCEA of only 0.06 ng/ml/6 hr for the 2-K-N plasma. The generated A II (Fig. 4) and the endogenous A II (Fig. 5) plasma concentrations for the same sample were 0.56 and 0.49 ng/ml, respectively.

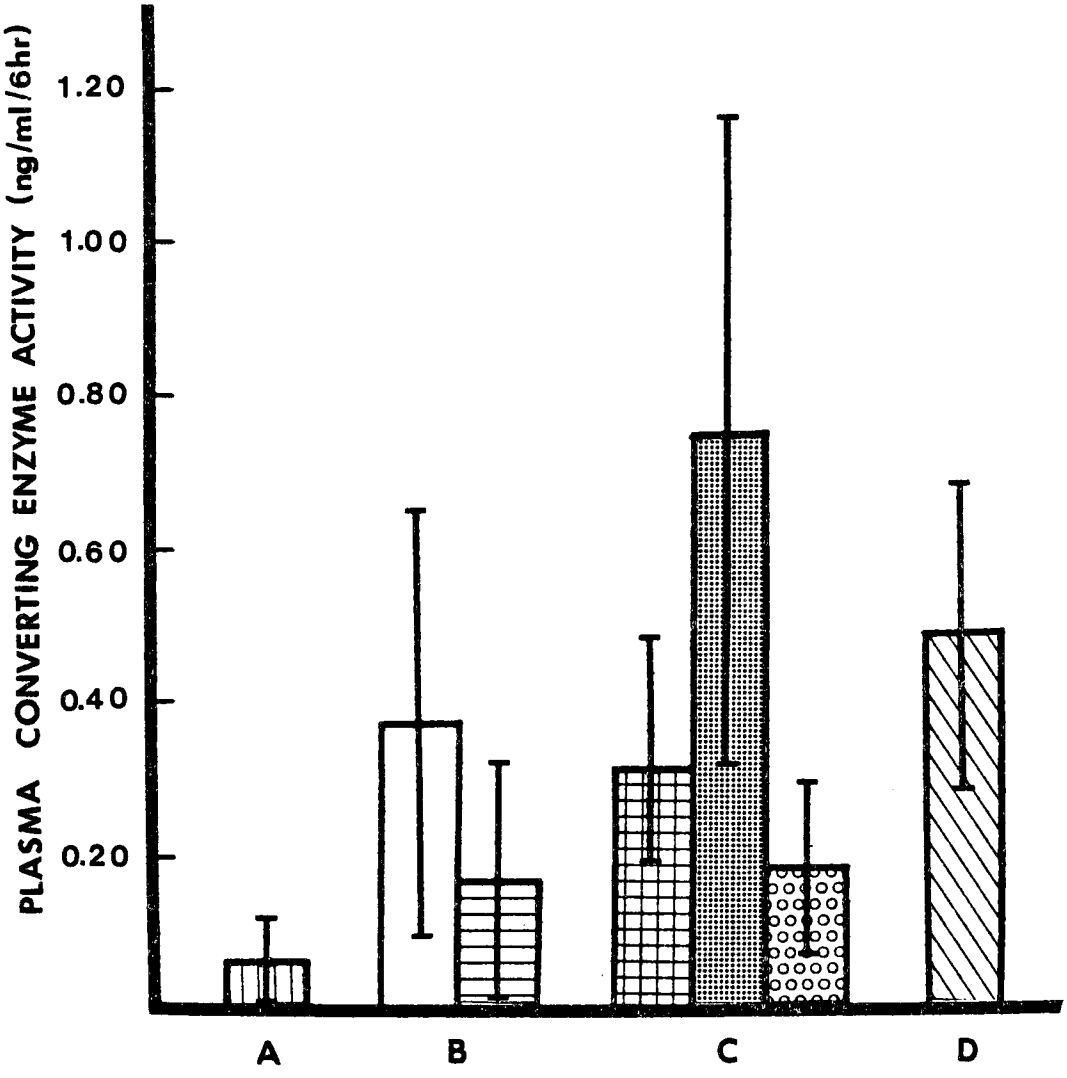

PLASMA CONVERTING ENZYME ACTIVITY (ng/ml/6hr)
1.20
1.00
0.80
0.60
0.40
0.20
A
B
C
D

Fig. 10. Generated A II concentrations of 10, 20, and 30-day kidney Goldblatt (1-K-G) and salt-induced experimental plasma samples, compared to their controls. Vertical bars represent the standard deviation (±) of the mean.

controls

1-K-G experimentals

salt-induced experimentals

value of 0.06 ng/ml/6 hr in the plasma samples of both the 10 and 20 day salt-induced hypertensive rats, to 0.18 ng/ml/6 hr in the 30-day experimentals (Fig. 9, Table 3). The values cited here show an increase of 200% in PCEA, but when compared to values of the controls and 1-K-G hypertensive rats, they become almost insignificant in the 10 and 20-day plasma samples (Table 3). Controls for this experimental model were the same animals used for the 1-K-G rats.

Similar activities for the malignant hypertensive model (5-HTP induced) are illustrated in Fig. 3 and Table 4. These results show a decrease in PCEA from 0.37 ng/ml/6 hr in the plasma of the controls to 0.16 ng/ml/6 hr in that of the 5-HTP experimentals. These values represent a decrease in PCEA of 131.2% in the 5-HTP experimentals when compared to the 5-HTP control animals.

Generated and endogenous A II plasma concentrations

The values obtained by radioimmunoassay determinations of A II concentrations in the experimental plasma samples after (generated A II) and before (endogenous A II) 37 C incubation for 1 hr are illustrated in Figs. 10 and 11, and in Tables 2, 3 and 4. Figures 10 and 11 demonstrate that the plasma of the control animals representing both the 1-K-G and the salt-induced hypertensive models show a decrease in both generated and endogenous A II concentrations from 10 to 30 days. These values show a 100% decrease, or from 1.86 ng/ml at 10 days to 0.93 ng/ml at 30 days. A 93% decrease in endogenous A II concentration was observed during the same time period. The latter values were from 1.18 to 0.61 ng/ml. In contrast, there was an increase in concentrations in the 1-K-G experimental plasma of 95.3% (from 1.06 to 2.07 ng/ml) and 118% (from 0.61 to 1.33 ng/ml),

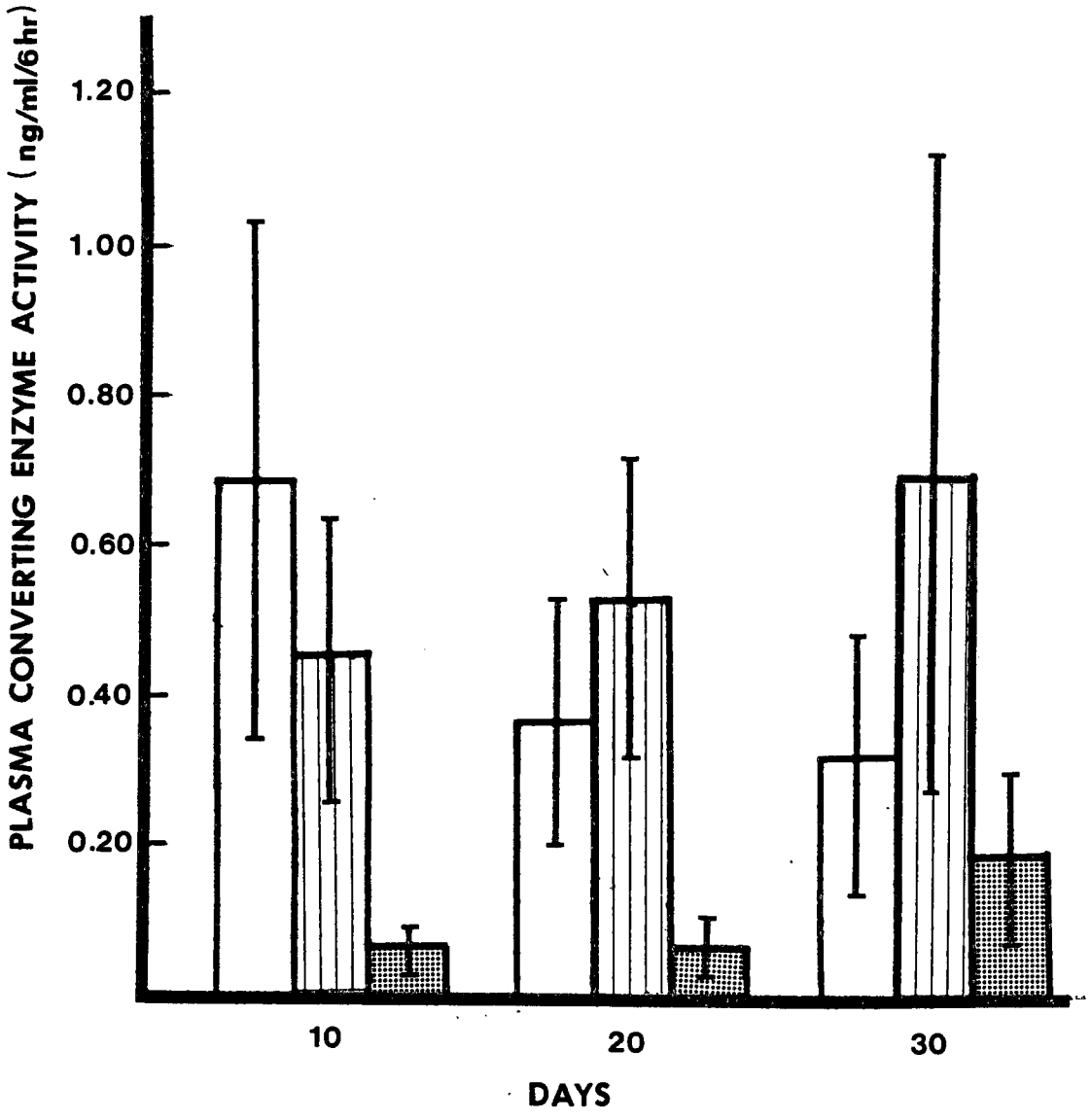

PLASMA CONVERTING ENZYME ACTIVITY (ng/ml/6hr)
1.20
1.00
0.80
0.60
0.40
0.20
10
20
30
DAYS

Fig. 9. Plasma converting enzyme activity (PCEA) of 10, 20, and 30-day one kidney Goldblatt (1-K-G) and salt-induced experimental plasma samples, compared to their controls. PCEA is expressed as a function of generated and endogenous A II ocncentrations. Vertical bars represent the standard deviation (±) of the mean.

controls

1-K-G experimentals

salt-induced experimentals

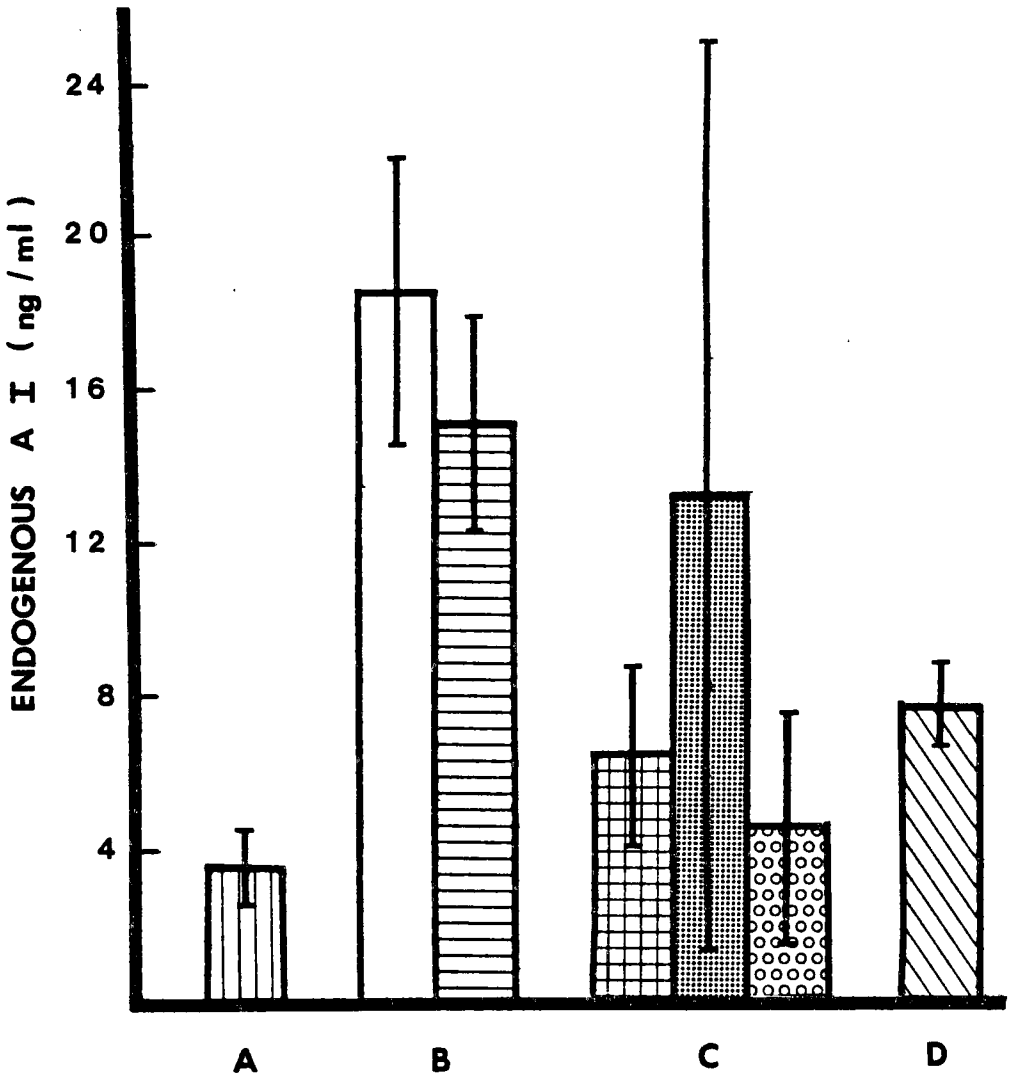
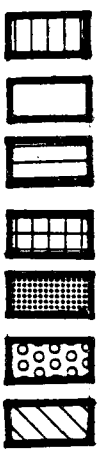

ENDOGENOUS A I (ng / ml)
24
20
16
12
8
4
A
B
C
D

Fig. 8. Endogenous A I concentrations of experimental plasma samples, compared to their controls, 36 hr two kidney nephrectomized (2-K-N) and spontaneously hypertensive rats (SHR's). Vertical bars represent the standard deviation (±) of the mean.



A. 36 hr 2-K-N

B. 5-HTP controls

5-HTP experimentals

C. 30-day controls

30-day 1-K-G experimentals

30-day salt-induced experimentals

D. SHR's

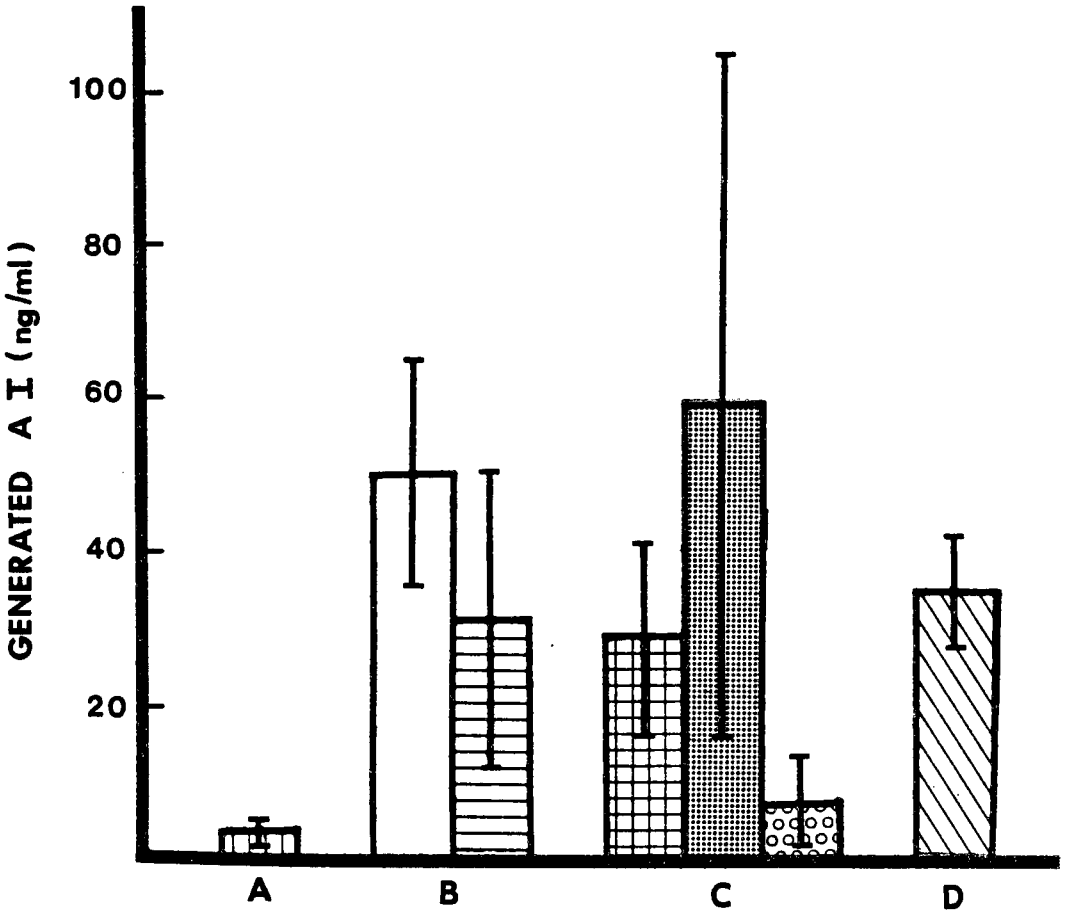

GENERATED A I (ng/ml)
100
80
60
40
20
A
B
C
D

Fig. 7. Generated A I concentrations of experimental plasma samples, compared to their controls, 36 hr two kidney nephrectomized (2-K-N) and spontaneously hypertensive rats (SHR's). Vertical bars represent the standard deviation (±) of the mean.

A. 36 hr 2-K-N

B. 5-HTP controls

5-HTP experimentals

C. 30-day controls

30-day 1-K-G experimentals

30-day salt-induced experimentals

D. SHR's

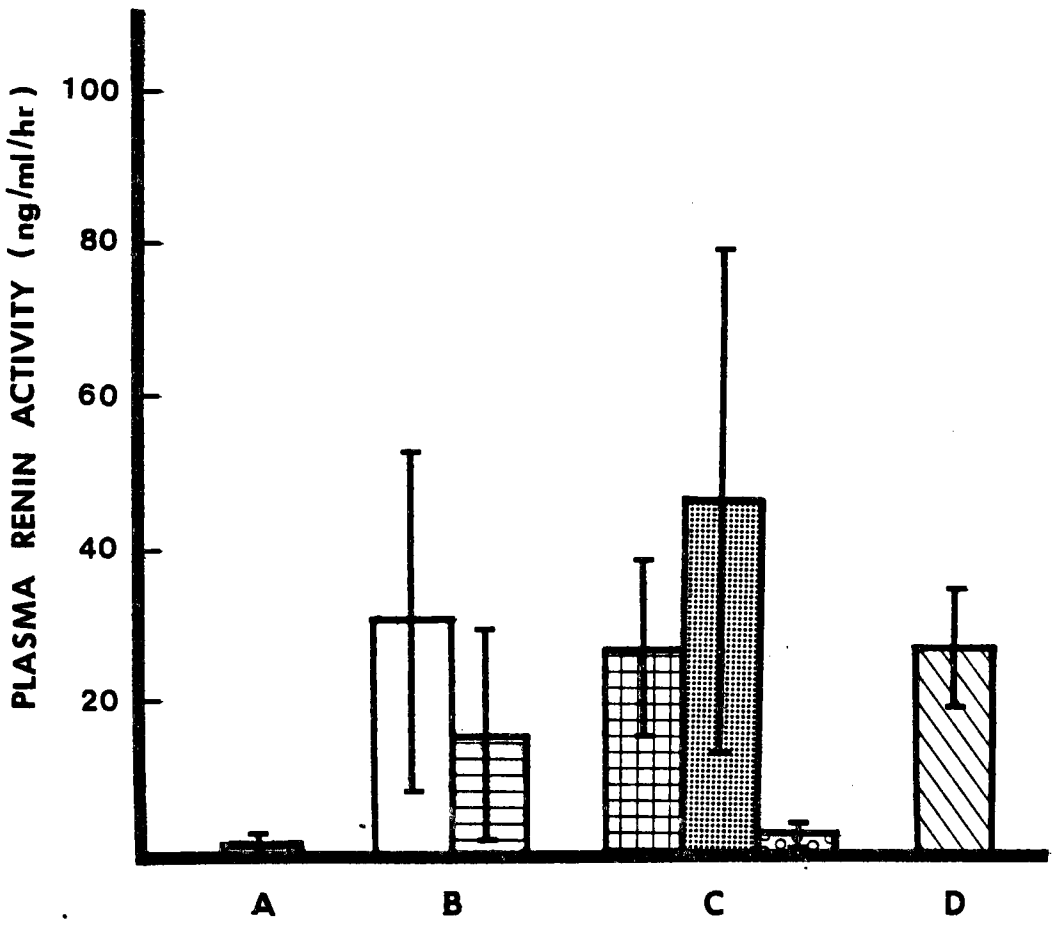
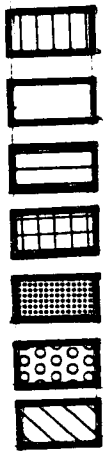

PLASMA RENIN ACTIVITY (ng/ml/hr)
100
80
60
40
20
A
B
C
D

Fig. 6. Plasma renin activity (PRA) of control and experimental plasma samples, expressed as a function of generated and endogenous A I concentrations. Vertical bars represent the standard deviation (±) of the mean.



A. 36 hr 2-K-N

B. 5-HTP controls

5-HTP experimentals

C. 30-day controls

30-day 1-K-G experimentals

30-day salt-induced experimentals

D. SHR's

In contrast, the same type of data compiled from radioimmunoassay determinations made simultaneously on plasma samples from a group of 13 SHR's reveal a PCEA of 0.48 ng/ml/6 hr (Fig. 3). The generated A II plasma concentration in this group of rats was 1.20 ng/ml (Fig. 4), while the endogenous A II was 0.71 ng/ml (Fig. 5). As is evident, the PCEA of this group represents an increase of approximately 700% above that for the 2-K-N rats.

Plasma renin activity (PRA) in the same plasma samples from the 2-K-N rats derived by similar computations of A I concentrations was 0.45 ng/ml/hr (Fig. 6). The generated A I concentration was 3.98 ng/ml (Fig. 7), while the endogenous A I was measured at 3.52 ng/ml (Fig. 8).

PRA in the SHR plasma was 27.08 ng/ml/hr. These data demonstrate that the PRA in the plasma of the SHR's was about 60 times greater than that in the 2-K-N plasma samples. At the same time it was observed that the concentration of generated A I in the SHR plasma sample was 34.39 ng/ml, compared to an endogenous concentration of 7.64 ng/ml.

Plasma converting enzyme activities in the experimental hypertensive models

The results referred to as PCEA were observed after radioimmunoassay determinations of A II concentrations in the plasma of the 3 experimental hypertensive models. The data obtained using modifications of the method of Poulsen (1971) are presented in Fig. 9 and Tables 2, 3, and 4. Fig. 9 and Table 2 show a 64.4% increase in PCEA in the 1-K-G rats from 10 to 30 days. In contrast, when these results are compared with the controls it can be seen that there is a decrease in PCEA of approximately 119% during the same time periods.

It was also observed that the PCEA increased only slightly from a low

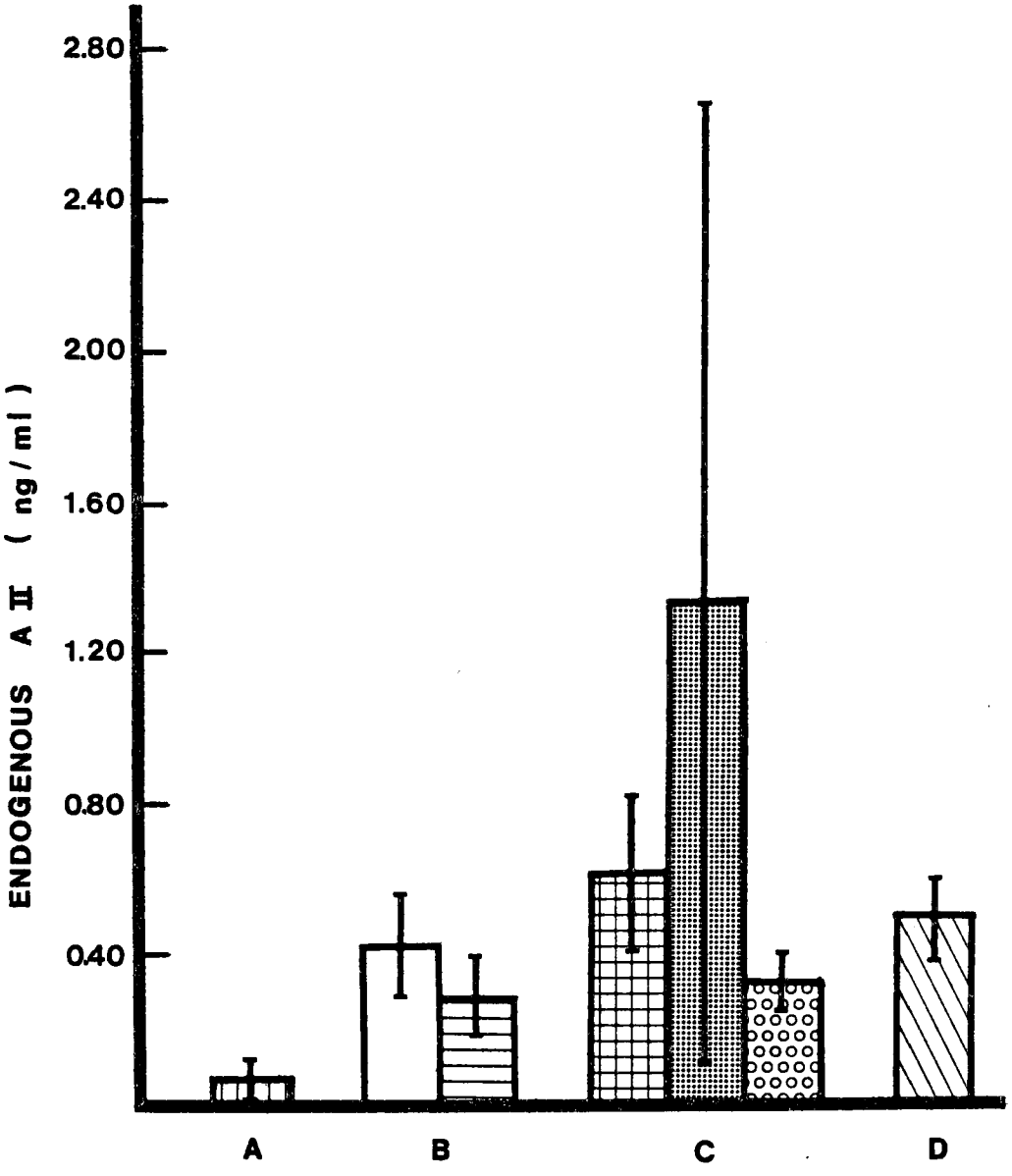
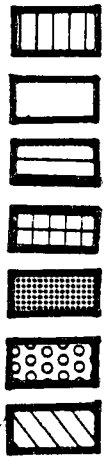

ENDOGENOUS A II (ng / ml)
2.80
2.40
2.00
1.60
1.20
0.80
0.40
A
B
C
D

Fig. 5. Endogenous A II concentrations of experimental plasma samples, compared to their controls, 36 hr two kidney nephrectomized (2-K-N) and spontaneously hypertensive rats (SHR's). Vertical bars represent the standard deviation (±) of the mean.



A. 36 hr 2-K-N

B. 5-HTP controls

5-HTP experimentals

C. 30-day controls

30-day 1-K-G experimentals

30-day salt-induced experimentals

D. SHR's

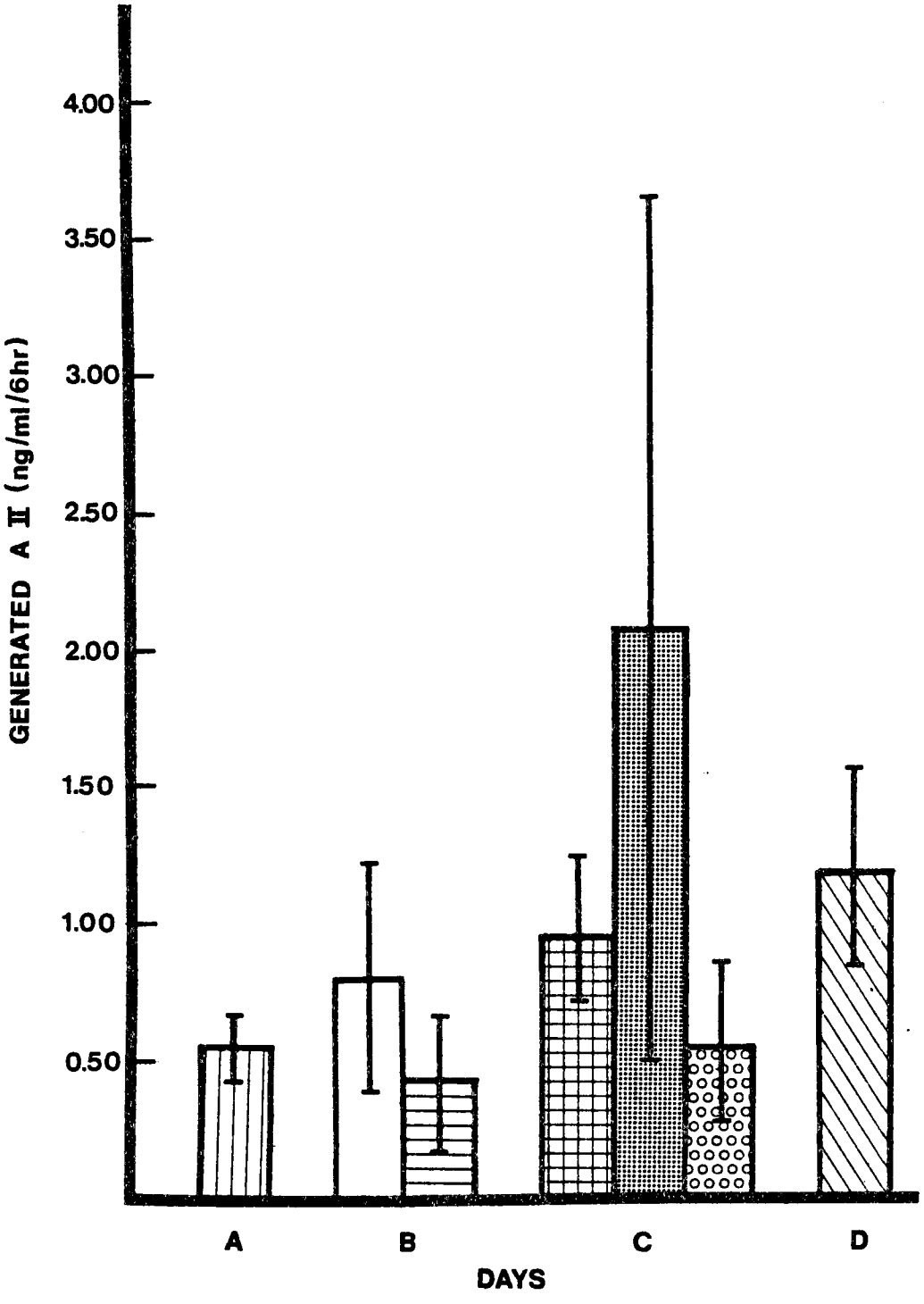
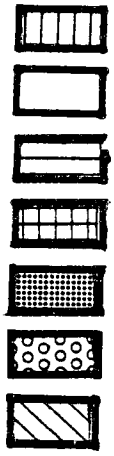

4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
GENERATED A II (ng/ml/6hr)
A
B
C
D
DAYS

Fig. 4. Generated A II concentrations of experimental plasma samples, compared to their controls, 36 hr two kidney nephrectomized (2-K-N) and spontaneously hypertensive rats (SHR's). Vertical bars represent the standard deviation (±) of the mean.



A. 36 hr 2-K-N

B. 5-HTP controls

5-HTP experimentals

C. 30-day controls

30-day 1-K-G experimentals

30-day salt-induced experimentals

D. SHR's

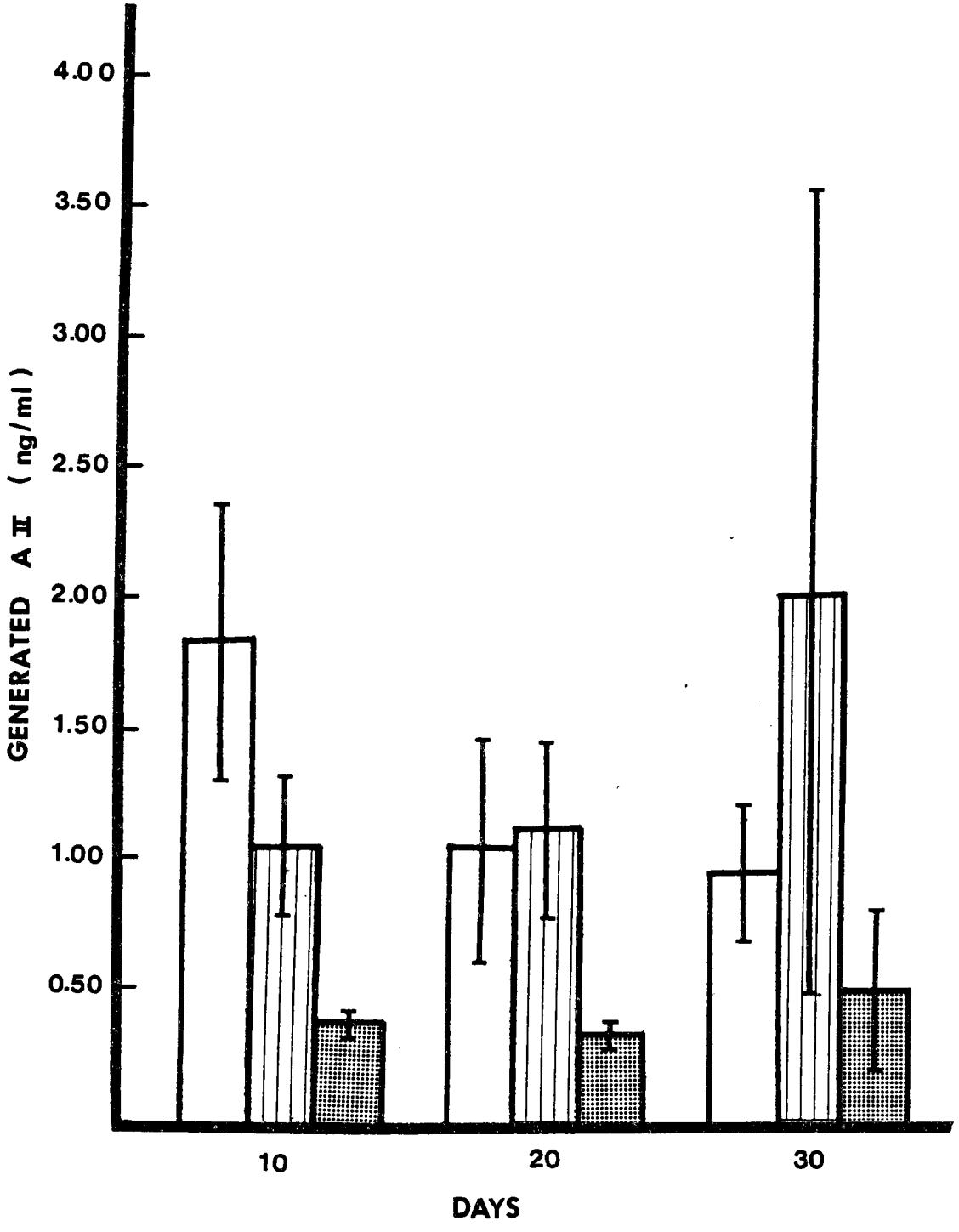
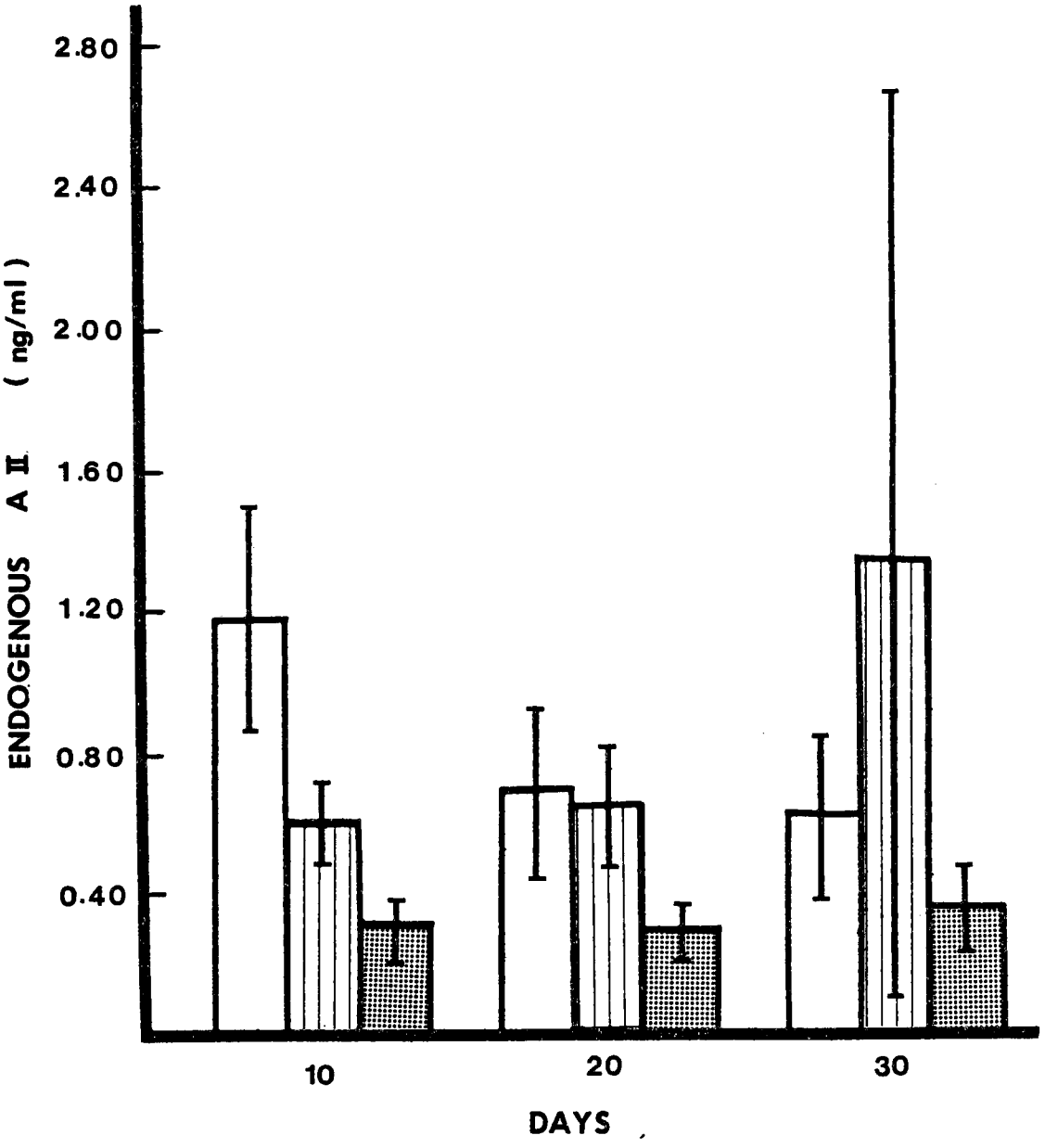

4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
GENERATED AII (ng/ml)
10
20
30
DAYS

Fig. 11. Endogenous A II concentrations of 10, 20, and 30-day one kidney Goldblatt (1-K-G) and salt-induced experimental plasma samples, compared to their controls. Vertical bars represent the standard deviation (±) of the mean.

controls

1-K-G experimentals

salt-induced experimentals


2.80
2.40
2.00
1.60
1.20
0.80
0.40
ENDOGENOUS A II (ng/ml)
10
20
30
DAYS

respectively, in generated and endogenous A II.

Plasma from the salt-induced rats also show increases in both concentrations. For the generated sample the increase was 42.1% (from 0.38 to 0.54 ng/ml). An increase of only 11.1% was noted in the endogenous A II samples from 10 to 30 days.

The 5-HTP plasma samples (figs. 7 and 8, and Table 4) show a decrease in generated and endogenous A II when compared to the control. The decrease in the generated sample was 79.5%, or from 0.79 ng/ml in the controls to 0.44 ng/ml in the 5-HTP experimentals. The endogenous A II plasma samples show a 50% decrease, or from 0.42 ng/ml in the controls to 0.28 ng/ml in the experimentals.

In the 36 hr 2-K-N plasma (Figs. 7 and 8, and Table 4) the concentration of generated A II was 0.56 ng/ml, while the endogenous concentration was 0.49 ng/ml. These values reveal a difference of only 0.06 ng/ml between the two concentrations. In contrast, the plasma of the SHR's show a generated concentration of 1.20 ng/ml compared to an endogenous concentration of 0.71 ng/ml. The latter values represent a difference of 0.49 ng/ml.

Plasma renin activity

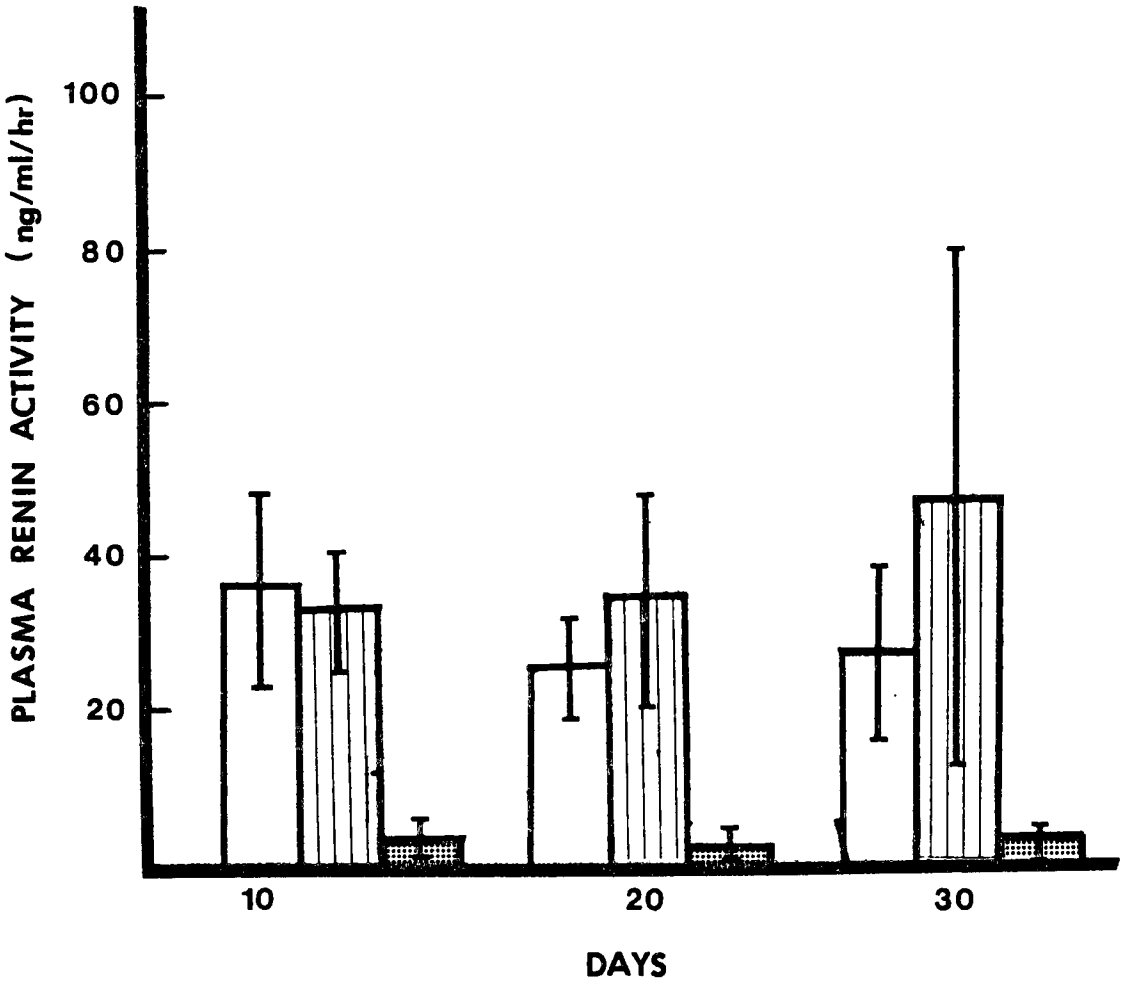
The experimental results illustrated in Fig. 12 show that the computed PRA (generated minus endogenous concentrations of A I) in the 1-K-G experimental rats increases approximately 40.7% or from 39.98 ng/ml/hr at 10 days, to 46.40 ng/ml/hr at the end of the 30-day experimental period. In comparison there was a 32.3% decrease in PRA in the control animals. The PRA values for the 10 and 30-day controls were 35.69 and 26.98 ng/ml/hr, respectively.

Fig. 12. Plasma renin activity (PRA) of 10, 20, and 30-day one kidney Goldblatt (1-K-G) and salt-induced experimental plasma samples, compared to their controls. PRA is expressed as a function of generated and endogenous A I concentrations. Vertical bars represent the standard deviation (±) of the mean.

controls

1-K-G experimentals

salt-induced experimentals


PLASMA RENIN ACTIVITY (ng/ml/hr)
100
80
60
40
20
10
20
30
DAYS

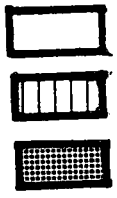
Very little PRA was detected in the plasma samples of the salt-induced hypertensive rats (Fig. 12). PRA in the 10, 20, and 30-day groups were 2.38, 1.51 and 2.26 ng/ml/hr in the order designated. These data show significant decreases in PRA in this model when compared to the controls and 1-K-G rats. PRA in the 5-HTP rats (Fig. 6) show a decrease from 30.65 ng/ml/hr in the controls to 15.33 ng/ml/hr in the experimentals. These values represent a 100% decrease in PRA for the 5-HTP induced hypertensive rats. Data pertaining to PRA in the 36 hr 2-K-N and SHR's have been reported in the results pertaining to the validity of the radioimmunoassay for the determination of A I and A II (Fig. 6 and Table 4).

Generated and endogenous A I concentrations

Figure 13 and Tables 2 and 3 show the computed data for the generated concentrations of A I determined by radioimmunoassay. These results show the differences between the control samples and that of 1-K-G and salt-induced experimentals collected at 10, 20 and 30 days. It is evident that there is a 72.8% decrease in generated A I, or from 57.77 ng/ml at 10 days to 33.42 ng/ml at 30 days in the control plasma samples. When compared to the data for the 1-K-G samples, the concentrations increase by 52.3%, or from 39.22 to 59.74 ng/ml during the same time periods.

From the salt-induced rats the generated A I values in the 10, 20 and 30-day plasma samples were 8.30, 5.27 and 6.68 ng/ml, respectively. These values show no significant differences at either time period. The data compiled for the 5-HTP samples reveal a 61% decrease in generated A I when compared to the controls. The measured concentrations were 49.08 ng/ml

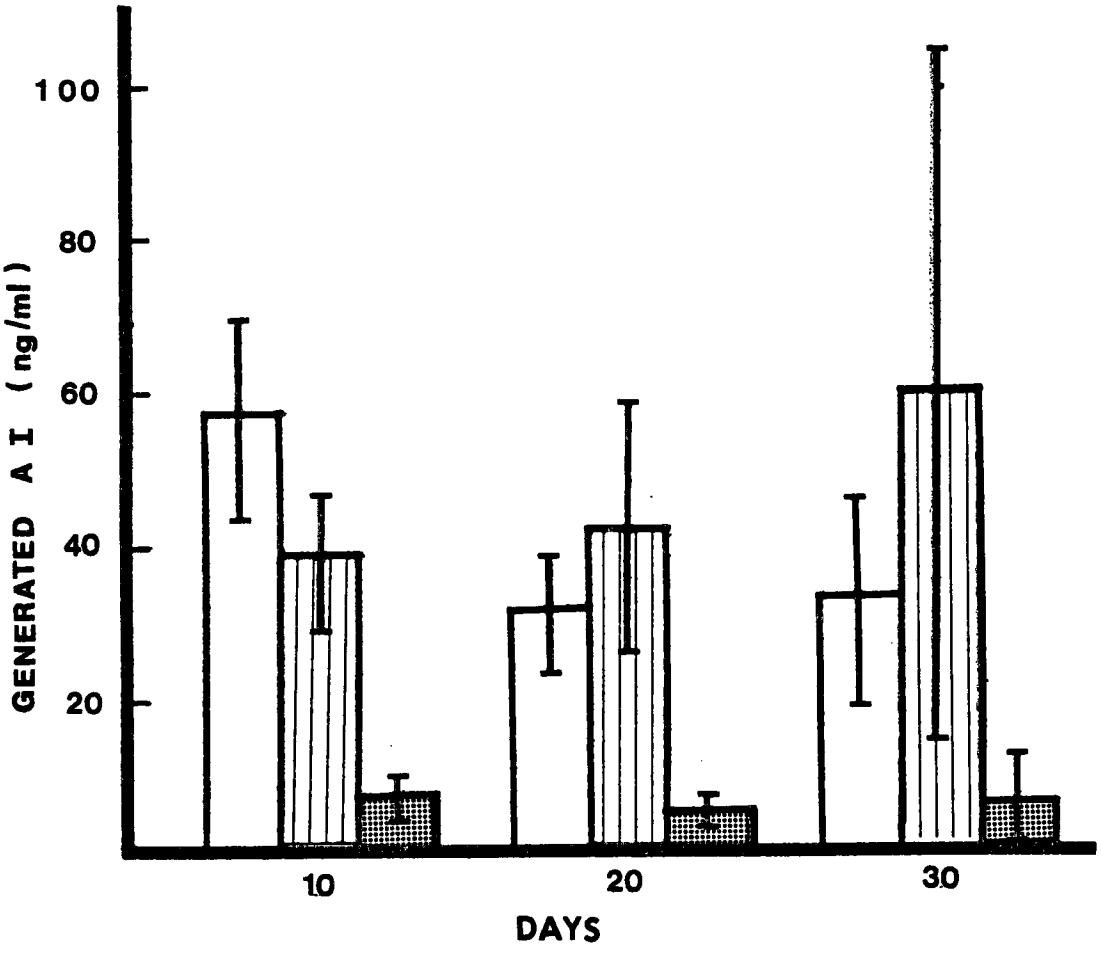
Fig. 13. Generated A I concentrations of 10, 20, and 30-day one kidney Goldblatt (1-K-G) and salt-induced experimental plasma samples, compared to their controls. Vertical bars represent the standard deviation (±) of the mean.



controls

1-K-G experimentals

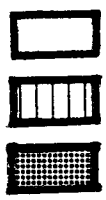
salt-induced experimentals


100
80
60
40
20
GENERATED AI (ng/ml)
10
20
30
DAYS

in the control samples and 30.47 ng/ml in the 5-HTP experimentals.

Endogenous A I concentrations decreased 243% in the control samples, but showed a 113.7% increase in the 1-K-G samples (Fig. 14 and Table 2). The concentration in the controls plasma was 22.07 ng/ml at 10 days and 6.44 ng/ml at 30 days. Values for the 1-K-G plasma were 6.24 and 13.34 ng/ml at the same time periods. Concentrations of endogenous A I in the plasma of the salt-induced animals were 5.92, 3.76, and 4.42 ng/ml at the end of the 10, 20, and 30-day time periods. These concentrations are not significantly different.

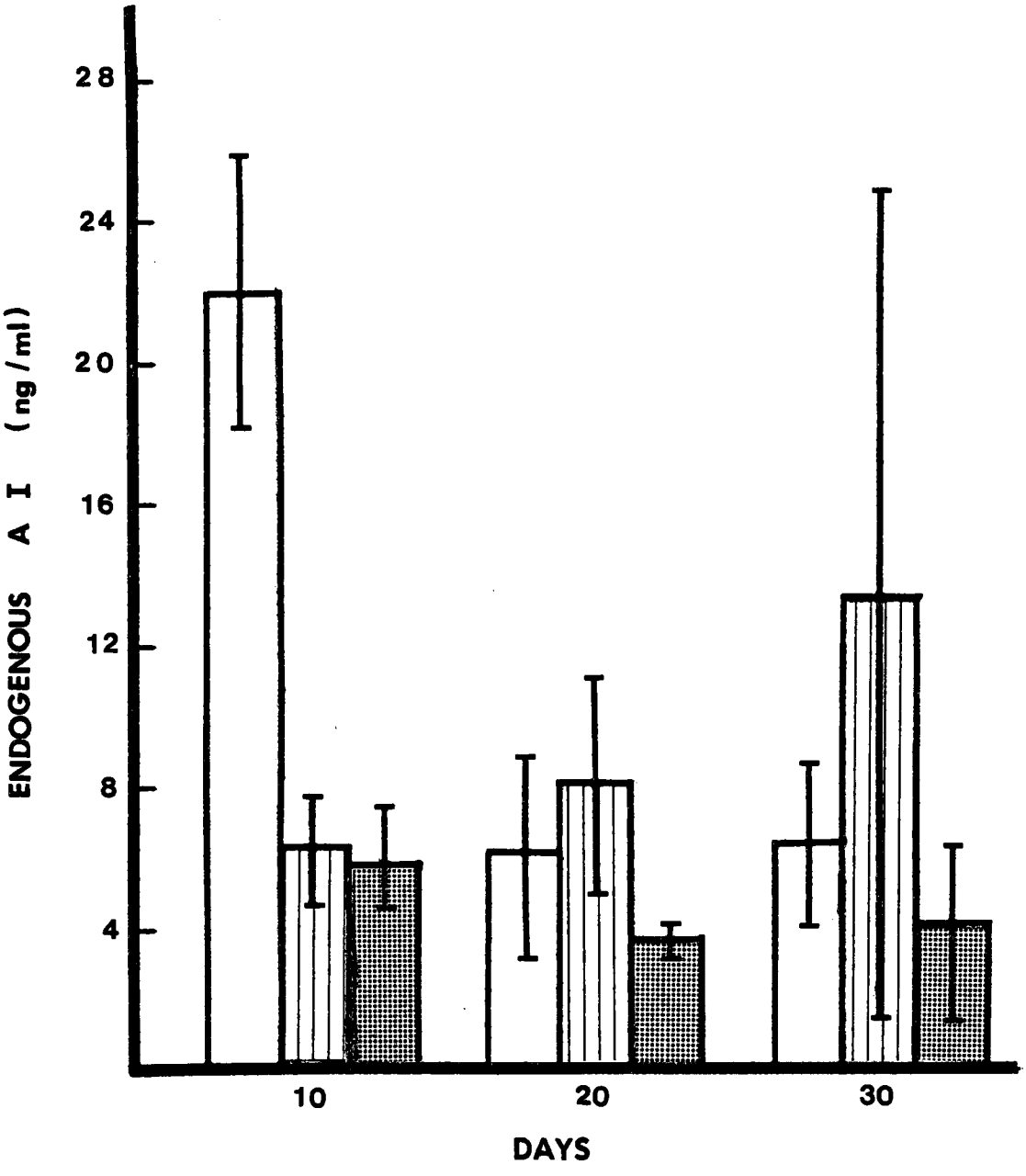
Fig. 14. Endogenous A I concentrations of 10, 20, and 30-day one kidney Goldblatt (1-K-G) and salt-induced experimental plasma samples, compared to their controls. Vertical bars represent the standard deviation (±) of the mean.



controls

1-K-G experimentals

salt-induced experimentals


28
24
20
16
12
8
4
ENDOGENOUS A I (ng/ml)
10
20
30
DAYS

CHAPTER V

DISCUSSION AND CONCLUSION

The recent flurry of interest in the converting enzyme was probably stimulated by two factors: the availability of a synthetic A I and the discovery that in the dog in vivo, conversion of circulating A I to A II takes place in the lungs (Ng and Vane, 1967, 1968) rather than in the blood. Since then, considerable controversy has developed over the degree of conversion of A I to A II, not only in the lungs, but in the plasma and other vascular beds.

These observations and the production of persistent hypertension in laboratory animals by experimental procedures, have made it possible to elucidate these and various other parameters of the renin dependent angiotensin system. Until recently, it had been assumed that all forms of hypertension could be explained by the same mechanism, and various investigators have used different animal models for the study of the effects of renin on blood pressure. However, observations of the actions of the angiotensins have led to the study of enzymes involved in the metabolism of the liberated peptides, including the A I converting enzymes.

The precise identification of the form of angiotensin recovered from in vitro incubation for plasma renin activity (PRA) determinations (A I, A II or a mixture) has become increasingly important due to chemical means for quantitation such as radioimmunoassay. Therefore, the possibility to discriminate A I and A II quantitatively in mixtures by such methods seemingly would provide means for a quantitative assessment of angiotensin plasma converting enzyme activity (PCEA). Thus, it became questionable

in our laboratory as to whether or not quantitative measurements of A I simultaneously with A II could be determined. Consequently, measurement of the ratio of A I to A II in plasma recovered as the 37 C incubation product by radioimmunoassay was used to examine PCEA.

The simplified radioimmunoassay method of Poulsen (1971) was used for studying PCEA. These procedures consisted of the capture of A II by use of an excess of A II antibodies with subsequent PCEA determinations. Methods previously available for measuring the activity of the converting enzyme have been the bioassay methods of Helmer (1957) using isolated strips of rabbit aorta, and of Bumpus et al. (1961) using isolated rat uterus, Huggins and Thampi's (1968) chromatographic separation, and Boucher et al. (1970) cation exchange adsorption technique. Many investigators have used these and other similar methods to study CEA, among them being Anderson (1967), Barrett and Sambhi (1969), and more recently Fitz et al. (1971). Only a few studies involving use of radioimmunoassay for CEA determinations have been reported. One such study in particular was that of Hollemans et al. (1969), cited because of its similarity to that used in this study, the difference was that in the latter study, a converting enzyme preparation from human lung was used for the identification of the incubation product of renin with subsequent radioimmunoassays for A I and A II.

The present study involved the use of plasma samples from experimentally induced hypertensive rats, in which simultaneous determinations of A I and A II concentrations were made with subsequent computations of PCEA. Validity of the radioimmunoassay procedures used were determined by the sensitivity of the assay to detect very low concentrations of A I

and A II in the plasma from rats that had their source of renin removed 36 hr prior to the collection of plasma samples. Plasma from 24 hr totally nephrectomized rats is known to show very little PRA due to removal of the kidneys which is the major source of plasma renin. Consequently, since the product of PRA is A I, which is also the substrate of the converting enzyme, the assays used should detect little or no A I or A II, and subsequently markedly reduced PCEA in the 36 hr 2-K-N plasma. PRA in the plasma samples from the 2-K-N rats examined were only 0.45 ng/ml/hr (± 0.25). These findings are in agreement with those of Lever and Robertson (1964) who found that PRA is markedly reduced or abolished within 24 hr of removal of both kidneys. In contrast to the reduced PRA detected in the 36 hr 2-K-N rats, the plasma from the SHR's that had both kidneys intact, showed a maximum PRA of 27.08 ng/ml/hr (± 7.4) at pH 7.4, the known optimal pH for renin substrate reaction. It would therefore be reasonable to expect the PCEA to be markedly reduced in the 2-K-N plasma (0.06 ng/ml/6 hr), and increased in the SHR's (0.48 ng/ml/hr). These results are also confirmed by those of Menard and Catt (1972). They observed that the specificity of a similar radioimmunoassay system for renin activity failed to detect A I after incubation of 500 µl of 24 hr nephrectomized rat plasma up to 6 hr at 37 C.

The acute rise in blood pressure observed at 30 days when a clip is placed around one artery (one kidney made ischemic) and the other removed, has been well established since its development by Goldblatt (1934). Many investigators have consequently used the technique to study hypertensive phenomena, and it is commonly agreed that such rises in blood pressure in some types of experimentally induced animals are accompanied

by either increases or decreases in renin related activities.

The knowledge that all forms of induced hypertensive animals are not supported by the same mechanism is therefore essential to understanding the different results obtained as concentrations of A I and A II determined in plasma samples. As these concentrations vary in the plasma of the different models or types of experimental hypertensive animals, apparently PRA, and subsequently PCEA, would also vary.

In the 1-K-G hypertensive rats, increases of 40.7 and 64.4%, respectively, in PRA and PCEA from 10 to 30 days were reported. There must be some correlation between PRA, PCEA, and blood pressure in this model. Prior to the operation, these rats had initial mean blood pressures of 100.4 mm Hg, with increases of from 128.9 (± 15.71) at 10 days to 169.3 (± 18.43) mm Hg at 30 days. All increases (PRA, PCEA and blood pressures) were significant ($P < 0.05$) when compared to the control animals at the 30-day period, because all of the latter animals showed decreases in the same parameters during the same time periods.

Blood pressures of 108.6, 110.9 and 106.2 mm Hg were reported as being observed in the control animals during the experimental period. These rats had only the right kidney removed, and as in the 1-K-G rats, received tap water to drink. PRA decreased from 35.69 at 10 days to 26.98 ng/ml/hr at 30 days. This decrease in PRA was approximately 33.2%, while at the same time a decrease of 64.4% in PCEA was reported for these rats (0.68 to 0.31 ng/ml/6 hr).

The chronic etiology or mechanisms that are probably responsible for the observed differences in blood pressure and all other parameters in the 1-K-G and their controls will not be discussed here. It may be

important though to re-emphasize at this time that the transitory changes in these animals must be borne by phenomena relative to the renin dependent angiotensin system. Blood pressures in the control animals remained relatively stable, but there were significant decreases in all parameters observed (PRA, PCEA, and A I and A II concentrations).

Prior to the development of radioimmunoassay in the late 1950's and 1960's, investigations relative to experimental hypertension involved use of bioassay procedures to determine the effects of renin, the converting enzyme and their products on various kinds of smooth or involuntary muscle tissue. Since its development, the procedure has been used mainly to determine A I and A II concentrations in plasma and other tissues in conjunction with PRA, PRC and PRS (Gocke et al., 1968; Poulsen, 1969b). Consequently, use of radioimmunoassay relative to the converting enzyme has dealt mainly with aspects of the development of methods for measurement of its activities, kinetics, inhibition and the sites of conversions of A I to A II. Therefore, the etiology of hypertension and parameters relative to the increases or decreases in PCEA and A II concentrations observed in the experimental models and their controls are further discussed in light of PRA and A I concentrations.

In furthur reference to the data obtained from radioimmunoassay determinations of the 1-K-G plasma, Lever and Robertson (1964) reported that during the first month of hypertension produced by constricting one renal artery and removing the opposite kidney, significantly higher renin levels are found. This confirms the present findings, discussed earlier. They are further substantiated by Carretero and Gross (1967) who observed that in experimental renal hypertension, increased renin production is

accompanied by an increased concentration of PRS. The present findings also correspond to observations in human hypertension by Helmer and Judson (1963) in which both renin and its substrate (angiotensinogen) were found to be elevated. Leenen and de Jong (1970, 1971) reported a 40% increase in PRA in venous blood from rats as early as day 1 upon application of a solid renal arterial clip. The increase was 40.7% at 30 days in the present study, but the plasma used was from a mixture of both venous and arterial blood.

It might be suggested that excessive renin release after renal artery constriction and removal of the opposite kidney served as the stimulus for the rise in blood pressure. This assumption is in agreement with Romero et al. (1970, 1973), however, these investigators suggest that a high renin level is not necessarily involved in the continued maintenance of hypertension. They theorized that renin secretion is the result of a rise in blood pressure after unilateral constriction due to a negative sodium balance. Their investigation was done using sheep with unilateral artery constriction, followed in four weeks by removal of the opposite kidney. These investigators assumed that when the first renal artery is constricted, there is a rise in blood pressure followed by a transient excessive sodium loss from the contralateral kidney, and that this may be a stimulus for renin release. Blair-West et al. (1968) have shown after similar experiments that such events accompany rises in blood pressure and may explain the transient increase in PRA when one artery is constricted in the sheep. In other experiments the latter investigators showed that if the blood pressure rose so as not to create a negative sodium balance or if the contralateral kidney had previously been removed

no increase in PRA was seen when the renal artery was clamped.

The present findings do not support the latter contention because opposing results were observed at 30 days when the left renal artery was constricted. For at the same time that the left artery was constricted, the right kidney was removed, and still, an increase was observed in PRA, PCEA, and A I and A II concentrations. Since these rats had as the sole functional kidney, one that had its renal artery constricted, it appears that other underlying mechanisms must have been responsible for the simultaneous increase in blood pressure, and the other renin dependent phenomena. Evidently, the development of 1-K-G hypertension cannot be explained simply by the action of one or more substances that may be released or produced in response to different stimuli, but is rather the result of multifunctional regulation. It appears that the constriction was the underlying factor responsible for the increased activities observed in this hypertensive model.

Opposing results observed in the controls for the 1-K-G model can more readily be interpreted as being in accord with the negative salt balance theory of Blair-West et al. (1968). These rats had only one kidney removed with no arterial constriction on the remaining kidney, and while such a transient increase in all activities existed at day 10, there was a tremendous decrease in all activities by the 30th day. Generated A I and A II in the control animals decreased by 72.8% and 100%, respectively, at 30 days. The concentrations at 10 days, 57.55 (A I) and and 1.86 ng/ml (A II), were approximately the same as those reported for the 1-K-G at 30 days, which were 59.74 and 2.07 ng/ml. PRA and PCEA values also paralleled each other at these respective time periods.

Therefore, it is reasonable to assume that a negative salt balance existed in the controls from the time of the removal of one kidney until the 10th day. Although there was no increase in blood pressure because of the loss of one kidney at the 10th day, a transitory decrease in all other activities was reported. The mechanisms underlying these observations may be due to homeostasis, wherein the remaining kidney was performing all functions previously performed by two, and thereby showed increases in all renin-dependent angiotensin activities. By the 20th and 30th day, wherein decreases in these activities were reported, it is assumed that homeostasis had been attained and that there was uniform physiological stability with the one remaining kidney. This reasoning is supported by the clinical observation that an animal can maintain physiological stability with only one kidney and not encounter hypertensive disorders.

Opposing results from that reported for the control rats have been presented in those given 1.5% saline as drinking water. Like the controls, these rats also had the right kidney removed. PCEA increased from a significantly low value of 0.06 ng/ml/6 hr at day 10 and 20 to 0.18 ng/ml/6 hr in the 30-day plasma. While this represents an increase in activity of approximately 200%, it is of importance to emphasize that these values are very low when compared to similar PCEA's in the controls and other experimental models.

If PCEA of the 10-day salt induced rats is compared to the same activities in the 10-day controls, a significant decrease of 1033.3% is seen. The decrease is not as pronounced in the 30-day animals; therefore, it must again be re-emphasized that there was a decrease in PCEA in the

control rats from 10 to 30 days. Furthermore, it is reasonable to expect that in 30 days, there would be some attempt by the one remaining kidney to overcome the effects of the chronic salt ingestion.

PRA was also reported as being significantly decreased at each time period, averaging only 2.05 ng/ml/hr for the 3 time intervals. At 10 days a decrease in PRA of approximately 140% was seen when these activities of the salt fed rats were compared with similar activities in the controls. Significantly lower concentrations of generated and endogenous A I and A II were also reported as being found in the plasma of the salt loaded rats.

Systolic blood pressure in these rats increased from approximately 88.9 mm Hg at the beginning of the experiment to 135.4 mm Hg at 30 days. The 30-day mean blood pressure does not appear to represent a chronic state of hypertension, since it has been reported in our laboratory that 150 mm Hg represents the hypertensive state. The highest blood pressure observed among 7 rats in the 30-day group was 152 mm Hg. In one rat, the blood pressure only reached 124 mm Hg by day 30. These are not unexpected results, for Dahl and Schackow (1964) reported that blood pressure responses to chronic salt feeding in rats may range from none to fulminating hypertension and even rapid death. They also reported that the hypertension may develop at different intervals after the onset of salt feeding. Once hypertension has appeared, it rarely ever disappears if salt feeding is continued. In our laboratory, blood pressures as high as 235 mm Hg have been observed in rats during chronic salt ingestion. Two rats from a beginning number of 9 in the 30-day group died during the course of this experiment.

Chronic salt ingestion apparently suppresses renin release from the renin secreting cells of the kidney, thereby cutting off the renin-angiotensin system. Carretero and Gross (1967) found that a high sodium diet in rats is followed by a pronounced reduction in the concentration of renin in the kidney and in plasma. If at the same time these rats are administered deoxycorticosterone acetate, renin may fall to levels too low to be estimated or even disappear completely. Moreover, Brown et al. (1964) reported that when sodium intake is restricted, the juxta-glomerular cells increase in size and granularity and that the extractable renin of the whole kidney is elevated. A diet rich in sodium produces the reverse effects. These ovservations, therefore, substantiate our findings of an approximate 140% decrease in PRA when these activities in the 10-day salt induced rats were compared to the controls.

Osteen et al. (1967) reported that the administration of serotonin (5-hydroxytamine), or its precursor, 5-hydroxytophan (5-HTP) to mature male rats is followed by severe renal cortical necrosis. Large doses of 5-HTP which caused as much as a ten-fold increase in the tissue serotonin of treated rats had previously been observed by Undenfriend et al. (1957). The pharmacologic effect of the precursor is believed to be primarily due to the serotonin formed therefrom (Freter et al., 1957). The effects of 5-HTP on renal cortical tissue resemble malignant hypertension or the cancerous state, in which there is a rapid degeneration of cells of the kidney cortex.

It was reported that a group of 13 rats was administered injections of 5-HTP simultaneously with injections of the hormone estradiol benzoate. Mean systolic blood pressure increased from 105.4 at the beginning of the

experiment to 164 mm Hg after 6 such injections 50 days later. Control rats for this group of experimental hypertensive animals that were given sham injections of 0.9% saline and 0.1 ml of sesame oil were reported as demonstrating no significant increase in blood pressure. PCEA was found to be significantly decreased (131.2%) from that of the controls when the experiment was terminated and the plasma assayed. PRA also decreased by approximately 100%. Generated and endogenous A I and A II concentrations were also reported as being decreased from those observed in the control animals.

Serotonin apparently controls the blood supply in the afferent vascular bed of the renal glomeruli, decreasing glomerular blood flow and filtration rate, and presumably also reducing the tubular blood supply (O'steen et al., 1969). It had previously been found that 5-HTP caused more severe renal lesions than did 5-HT, which suggested that the former might have a direct nephrotoxic effect.

Interestingly, the latter investigators have observed that estradiol benzoate in dosages devoid of any detectable effects on kidney structure or blood pressure enhances the effect of 5-HTP on both in either sex. Presumably, the hormone exerts its effect on blood pressure by augmenting the effect of 5-HTP upon those portions of the kidney critical to the development of high blood pressure. Large doses of estradiol chronically administered are believed to have some nephrotoxic action. Since estrogen (estradiol benzoate) is a naturally occurring hormone and 5-HTP is the precursor of the naturally occurring hormone, 5-HT, it was of interest to determine whether a combination of the two substances could be employed to cause elevated blood pressure.

Previously, resulting data have been discussed in light of the combined effects of the two hormones on the components of the renin-

angiotensin system. Despite the considerable body of evidence implicating disturbed kidney function in the etiology of certain forms of hypertension, the association is by no means invariable. When renal alterations and hypertsnsion coexist, it is not always possible to establish the cause and effect relationship between them. Therefore, there exists a possible relationship between them and renal enzymatic functions. If such were the case, it is at least conceivable that in certain clinical circumstances the components essential to some forms of human hypertensive disease could arise as a result of disturbed endocrine balance involving these specific hormones.

Of the various procedures for producing hypertension in animals, the simplest and most instructive has perhaps proved to be the original method of Goldblatt et al. (1934) using constriction of the renal artery. This method has the great advantage that structural damage to the renal parenchyma can be avoided. Therefore, most of the original experiments investigating the renin-angiotensin system has been based on this technique.

Indirect evidence of increased adrenocortical activity of the kidney after occlusion (constriction) of the renal artery has been obtained by enzymatic (PRA and PCEA) studies in plasma. When hypertension is produced by constricting one artery in the rat, there is apparently an increase in renin release based upon observation of increased PRA which has been presented herein. Activities of the renal enzyme (renin) ultimately lead to increased or decreased activities of the converting enzyme in plasma. The latter activities subsequently affect the concentrations of A II determined by radioimmunoassay procedures.

Decreases in these activities have been observed after chronic salt

ingestion. Similar effects have been obtained also after the administration of the serotonin hormone precursor, 5-HTP in rats, which causes proximal tubular necrosis and gross changes in the renal cortex (O'steen et al., 1967). It seems reasonable, therefore, to conclude that the adrenal cortex is in some way apparently affected by such stimuli to increased or decreased activities.

While these observations provide evidence for a direct renal-adrenocortical interaction in experimental hypertension, the question still remains whether this interaction actually causes the blood pressure to rise. If it does, what are the mode of operation and the site or sites of action in the cardiovascular system? The most obvious manner in which adrenal corticoids might act is by water or sodium retention associated with redistribution of tissue electrolytes, which in turn might modify vascular resistance or reactivity. The evidence for such changes in experimental hypertension is inconclusive.

The discovery of the renin-angiotensin system and the similarity between its hemodynamic effects and those of arterial hypertension appeared to provide a satisfactory basis for excessive vasoconstriction by A II. Increased renin production by the damaged kidney has been postulated as the cause of renal hypertension. Unfortunately, proof of this attractive hypothesis is still lacking.

The relevance of these various observations in laboratory animals to hypertension in man is not entirely a matter for speculation. The common features of human hypertension due to unilateral stenosis of the renal artery, and high blood pressure in the rat due to renal artery constriction, provide a sufficiently close parallel for the latter to be

accepted as one of the most dependable experimental models for studying the pathogenesis of renal hypertension in man. Much less certain is the application of animal studies to essential hypertension. In the late stages of essential hypertension, it might be possible that a renal factor is superadded when renal vascular lesions or necrosis develop. As has been demonstrated here by the use of 5-HTP, there is experimental support for this. Further evidence has been provided by the demonstration of increased secretion of aldosterone in malignant hypertension by many investigators. It is a fair presumption that a closer understanding of the pathogenesis of arterial hypertension in man will only be achieved by a clearer definition of factors underlying the physiological regulation of blood pressure.

CHAPTER VI

SUMMARY

The ability to quantitate A I and A II in mixtures of the peptides such as is found in plasma provides methods that can be used to examine plasma converting enzyme activities (PCEA). Therefore, measurements of the ratio of A I to A II recovered as the 37 C incubation product in plasma by radioimmunoassay determinations has been used to investigate this renin-angiotensin dependent phenomenon. The antibody capture technique was used for the determination of A II concentrations in plasma from 3 experimentally induced hypertensive models of male Sprague-Dawley rats.

1-Kidney Goldblatt Model

1. As blood pressure increased in these rats that had the left renal artery constricted and the right kidney removed, an increase in plasma renin activity (PRA) was accompanied by an increase in PCEA. Increases in these enzymatic activities, apparently caused by the arterial constriction, are assumed to be due to a negative sodium balance (sodium depletion).

2. Arterial constriction of the only functional kidney resulted in increased renal enzymatic activities. The concentration of A I produced reflects the subsequent PCEA with A II liberation.

3. The control rats for this model revealed no increase in blood pressure during the experimental period, but both PRA and PCEA were increased at 10 days. Significant decreases of 33.2% and 64.4% respectively in these activities were observed 20 days later. These observations in

the controls indicate that some animals with one functional kidney can maintain homeostasis and thereby regulate blood pressure.

Salt Induced Hypertensive Model

4. Due to chronic salt ingestion PRA was significantly suppressed in the plasma of the salt fed rats. Subsequently, as A I concentrations decreased, PCEA also decreased. These rats were given 1.5% saline as drinking water for 30 days after removal of the right kidney. Therefore, it is assumed that salt feeding suppresses renin release from the renin secreting cells of the kidney. This phenomenon results in cutting off the renin-angiotensin system.

5-hydroxytryptophan Induced Model

5. Administration of the serotonin hormone precursor, 5-HTP, simultaneously with the hormone estradiol benzoate resulted in reduced PRA and PCEA in the plasma of the experimental rats when compared to their controls.

6. Large doses of 5-HTP apparently had nephrotoxic effects on the kidney due to the serotonin hormone formed therefrom. This hormone is believed to cause rapid degeneration of kidney cortical cells which resemble malignant hypertension. This condition apparently resulted in reduced tubular blood supply, and subsequently decreased renal enzymatic activities.

7. Estradiol benzoate, when given in moderate dosages, is believed to have no detectable effects on kidney structure or blood pressure. It is assumed therefore, to enhance the effects of 5-HTP. Presumably, this hormone exerts its effects upon those portions of the kidney critical to the development of hypertension.

8. Mean systolic blood pressure increased significantly in rats injected with 5-HTP and estradiol benzoate over that of the controls that were given sham injections (ip) of 0.9% saline and 0.1 ml of sesame oil (sc).

Validity of the Radioimmunoassay for the Determination of A I and A II Concentrations

9. Thirty-six hr 2-kidney nephrectomized (2-K-N) rat plasma showed markedly reduced levels of PRA, with subsequent decreases in PCEA. These findings are apparently due to the removal of the source of the renal enzyme, renin.

10. Reduced PRA activities resulted in decreased concentrations of A I, the substrate of the converting enzyme. Decreased PCEA consequently resulted in a reduction of A II, the vasoconstrictor hormone of the renin-angiotensin system.

11. PRA was 60 times greater in the plasma of the spontaneously hypertensive rats (SHR), while PCEA increased by approximately 700% over that found in the 2-K-N plasma.

12. Results observed 36 hr after total nephrectomy, and in plasma from SHR's imply that the radioimmunoassay is a sensitive method and can be employed in the determination of PCEA. Apparently PCEA is a better index for studying experimental hypertension in laboratory animals than PRA studies, since its action is the last step in the renin-angiotensin system prior to the liberation of A II.

Radioimmunoassay of A II has been employed for the measurement of PCEA in rats and may provide a sensitive method for the assay of converting enzyme parameters in human beings. Moreover, there is increasing

evidence for the necessity of simultaneous measurement of PRA, PCEA and A I and A II during clinical studies on the renin-angiotensin system. The ability to perform such measurements in blood samples from small animals such as the rat also provide a valuable advantage, particularly during studies which require accurate evaluation of serial changes in the individual components of the renin-angiotensin system.

Finally, since A II is a more potent vassopressor agent than A I, measurements of A II generated in vitro may be underestimated when measured against A I.

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